# REGULATION OF LYMPHOCYTE FUNCTION BY SHEEP ENDOMETRIAL SERPIN-LIKE PROTEINS

By

WEN-JUN LIU

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## ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| AEBSF | 4-(2-aminoethy)-benzenesulfonyl floride hydrochloride |
| Ag | Antigen |
| ANOVA | Analysis of variance |
| AT | Antitrypsin |
| BSA | Bovine serum albumin |
| CD | Cluster differentiation |
| CM | Carboxymethyl |
| Con A | Concanavalin-A |
| cpm | Count per minute |
| $^{51}Cr$ | Chromium-51 |
| CTL | Cytotoxic lymphocyte |
| d | Day |
| dpm | Disintigrations per minute |
| DPPIV | Dipeptidyl peptidase IV |
| FACs | Fluorescence activated cell sorting |
| FITC | Fluorescein isothiocyanate |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| IEL | Intraepithelial lymphocyte |
| IFN | Interferon |
| IL | Interleukin |
| IU | International unit |
| kDa | Kilodalton |
| LAK | Lymphokine activated killer |
| M | Molar |
| mAb | Monoclonal antibody |
| mg | Milligram |
| MHC | Major histocompatibility complex |
| min | Minute |
| ml | Milliliter |
| MLR | Mixed leukocyte reaction |
| MW | Molecular weigh |
| NK | Natural killer |
| OSA | Ovine serum albumin |
| OVA | Ovalbumin |
| PAGE | Polyacrylamide gel electrophoresis |
| PBL | Peripheral blood lymphocyte |

| | |
|---|---|
| PE | Phycoerythrin |
| PHA | Phytohemagglutinin-L |
| pI | Isoelectric point |
| PMA | Phorbol myristate acetate |
| SDS | Sodium dodecyl sulfate |
| SCID | Severe combined immunodeficiency disease |
| SEC | Serpin enzyme complex |
| SECR | Serpin enzyme complex receptor |
| SEM | Standard error of the mean |
| serpin | Serine protease inhibitor |
| TCM | Tissue culture medium |
| TCR | T cell receptor |
| TGF | Transforming growth factor |
| Th | T helper cells |
| TNF | Tumor necrosis factor |
| U | Unit |
| UTM | Uterine milk |
| wk | Week |

## PRINCIPAL FEATURES OF LEUKOCYTE MARKERS MENTIONED IN THE TEXT*

| Markers | Molecular Structure | Main Distribution | Ligand or Proposed Functions |
|---|---|---|---|
| CD1 | $\beta_2$ microglobulin associated | Thymocytes, dendritic cells | Ligand for some $\gamma\delta$ T cells? |
| CD 3 | Composed of five chains($\gamma,\delta,\epsilon,\zeta/\zeta,\zeta/\eta$ | T cells | Signal transduction as a result of antigen recognition by T cells or lectin binding |
| CD4 | 55 kDa | Class II MHC-restricted T cells | Bind to class II MHC |
| CD8 | Composed of two 34 kDa chains; expressed as $\alpha\alpha$ or $\alpha\beta$ | Class I MHC-estricted T cells | Bind to class I MHC |
| CD2 | 50-70 kDa | NK cells, granulocytes, macrophages | Activation of NK cells, Antibody dependent cytotoxicity |
| CD2 | IL-2 receptor $\alpha$ subunit; 55 kDa | Activated T and B cells; macrophages | with $\beta$ and $\gamma$ of IL-2 receptor to trigger signal transduction |
| CD26 | 110 kDa | Activated T and B cells | Serine protease, costimulation of lymphocyte activation |
| CD29 | 130 kDa | Broad | Adhesion to extracellular matrix proteins, cell-cell adhesion. |
| CD30 | 105 kDa | Activated T cells and B cells | member of TNF family |
| CD44 | 80->100 kDa, highly glycosylated | Leukocytes | T cell activation; homing receptor for matrix components (e.g. hyaluronate) |
| CD45 | 180-200 kDa | Leukocytes | Role in signal transduction |
| CD45R | CD45RO:180 kDa CD45RA:220 kDa | CD45RO:memory T cells CD45RA:naive T cells | See CD45 |
| CD56 | Heterodimer of 135 and 220 kDa chains | NK cells | Homotypic adhesion |
| CD62L or L-selectin | 75-80 kDa | T cells and other leukocytes | Leukocyte-endothelial adhesion |
| CD69 | Homodimer of 28-34 kDa chains, phosphorylated glycoproteins | Activated B and T cells, NK cells | ? |

* Adapted and modified from Leukocyte typing IV. White Cell Differentiation Antigens, by Knapp W, Borken B, Gilks WR, et al. (eds.). Oxford, Oxford University Press, 1989.

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By

WEN-JUN LIU

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During pregnancy, immunoregulation is required to prevent uterine $\gamma\delta$ $TCR^+$ cells and natural killer (NK) cells which could be activated by trophoblast from damaging the conceptus. The sheep was used as a model to understand the role of progesterone-induced immunosuppressive proteins in regulation of the maternal immune system. Progesterone is a pregnancy-associated hormone that has immunoregulatory function and induces a pair of related endometrial serpin-like proteins called the uterine milk proteins (UTM-proteins) that may be important mediators of the effects of progesterone on immune functions. The effects of UTM-proteins on NK cell function were determined using the $Cr^{51}$ release assay. The proteins inhibited sheep and mouse NK cell activity and activation induced by

poly(I).poly(C) in vitro and in vivo, and prevented fetal loss in mice caused by injection of poly(I).poly(C). The mechanisms by which UTM-proteins regulated lymphocyte functions were studied. CD26 is a serine proteinase that costimulates T cell activation and is abundant in the uterine fluid. However, UTM-proteins did not reduce CD26 function as determined by enzymatic activity or costimulation of PHA-induced lymphocyte proliferation, indicating effects of UTM-proteins and CD26 on lymphocyte function are independent of each other. Also, specific binding of UTM-proteins to lymphocytes was demonstrated: Heparin reduced the binding of UTM-proteins to lymphocytes and inhibitory effects of UTM-proteins on PHA-induced lymphocyte proliferation. The lymphocyte-inhibitory effects of UTM-proteins were not mediated by blocking expression of IL-2 receptor or IL-2-induced activation. UTM-proteins did block activation of lymphocytes induced by PMA, indicating the proteins act at a site distal to protein kinas C activation. Finally, it was found that uterine $\gamma\delta$ $TCR^+$ cells were apparently activated by the local presence of the conceptus despite the presence of immunosuppressive UTM-proteins.

# CHAPTER I
# INTRODUCTION

The trophoblast is a potential allogeneic transplant that lies in direct contact with maternal immune cells but which subverts normal maternal immune responses (Medawar, 1953). One of the possible ways in which the trophoblast escapes maternal immune attack is through immunosuppression by locally-produced immunoregulatory molecules that modulate uterine lymphocyte activation (Billingham, 1964; Golander et al., 1981; Chaouat, 1987). Progesterone is a pregnancy-associated hormone that plays an important role as an uterine immunoregulator (Stites and Siiteri, 1983). In sheep, progesterone treatment prevents rejection of skin grafts in the uterus (Hansen et al., 1986) and reduces numbers of endometrial $CD45R^+$ lymphocytes (Gottshall and Hansen, 1993) and inhibits lymphocyte proliferation (Low and Hansen, 1988; Monterroso and Hansen, 1993). In addition, progesterone can induce secretion of a pair of endometrial serpin-like proteins, called the uterine milk (UTM)-proteins, that exert lymphocyte inhibitory activity (Segerson et al., 1984; Zhang and Miller, 1989; Skopets and Hansen, 1993). These proteins are the most abundant proteins present in uterine secretions of progesterone-treated and pregnant ewes (Hansen et al., 1987; Ing et al., 1989). Hence, it is proposed that inhibitory effects of progesterone on uterine immune functions are mediated in large part by

serpin-like proteins produced by the endometrium.

The sheep is an ideal model to test the role of progesterone in uterine immune function. First, progesterone-induced UTM-proteins have a wide range of functions that potentially regulate lymphocyte activation and proliferation. Second, changes in numbers of subpopulations of uterine lymphocytes in response to pregnancy are similar to changes in humans and mice (Lee et al., 1988; King and Loke, 1991; Heyborne et al., 1992; Meeusen et al., 1993; Suzuki et al.,1995). For example, $\gamma\delta$ T cell receptor $(TCR)^+$ cells in the endometrial epithelium are low during early pregnancy, but increase in number and activation during mid- and late pregnancy (Meeusen et al., 1993). The function of activated ovine $\gamma\delta$ $TCR^+$ cells is unknown but these cells could either produce cytokines beneficial to trophoblast or exert an anti-trophoblast cytotoxic response effect that is detrimental to conceptus. Therefore, it is important to determine whether the conceptus induces activation of endometrial intraepithelial $\gamma\delta$ $TCR^+$ cells and the action of UTM-proteins on activation of these lymphocytes. Third, the sheep uterus produces large quantities of UTM-proteins which makes purification and study of their fucntion and regulation simple.

The central goal of this dissertation was to characterize the immunoregulatory properties of UTM-proteins. Specific objectives were to study the mechanisms by which UTM-proteins regulate lymphocyte proliferation and activation; to determine effects of UTM-proteins on NK cell activity and activation; to study interaction between UTM-proteins and CD26 (a serine proteinase) on PHA-induced lymphocyte

proliferation; and to evaluate local and systemic effects of pregnancy on activation of uterine $\gamma\delta$ $TCR^+$ lymphocytes.

# CHAPTER II
# REVIEW OF LITERATURE

Maternal acceptance of the fetal allograft has often been called an immunological enigma (Gill, 1985). The pregnant uterus normally allows implantation and growth of fetal tissues even though the conceptus, which expresses both maternal and paternal-derived major histocompatibility complex (MHC) antigens, can be considered as an allograft with respect to the mother when mating occurs between genetically non-identical individuals (Medawar, 1953). The uterine endometrium contains a large of number of lymphocytes in mice, humans and sheep during pregnancy (Bulmer et al., 1987; Starkey et al., 1988; King and Loke, 1991; Nishikawa et al., 1991; Meeusen et al., 1991). Skin grafts placed in the uterus are readily rejected in rats (Head and Billingham, 1986) and sheep (Hansen et al., 1986). Therefore, the potential exists for the conceptus to trigger maternal immune responses to alloantigens expressed on the trophoblast. About one-third of human pregnancies end in spontaneous abortion (Wilcox et al., 1988). There is a lot of early pregnancy loss (about 40%) in sheep and cattle (Roberts et al., 1990; Thatcher et al., 1994). Understanding the contribution of immunological causes to these losses will provide the opportunity for diagnosis and treatment of pregnancy failure.

Maternal immune responses to trophoblast can include natural killer cell-

mediated natural immunity, cell-mediated immunity and humoral immunity (Smith, 1982; Toder et al., 1982; Wood, 1994). Despite the potential for its destruction, the conceptus is usually not rejected during normal pregnancy. The phenomenon by which an antigenically foreign conceptus is able to flourish in the uterus has intrigued biologists since Medawar first described this problem in 1953. Advances in immunology, biochemistry, molecular biology and cell biology have resulted in an increase in understanding of mechanisms which regulate the interaction between the maternal immune system and the conceptus. For example, recently, a T cell receptor (TCR) transgenic mouse model was used to confirm that maternal T cells specifically recognize paternal MHC class antigens during pregnancy (Tafuri et al., 1995). Concepts including immunosuppression, MHC gene expression and the "missing self" hypothesis, placental immunotrophism and redirection of cytokine production have been proposed as mechanisms by which the conceptus escapes maternal immunological attack. This literature review will first describe these theories as based primarily on the human and mouse research models. Next, concepts from these species will be compared to ideas derived using the sheep model.

## Maternal Immune Responses to the Conceptus

### The Conceptus as an Allograft

In outbred species, inheritance of paternal MHC antigens by the conceptus results in genetic mismatches to the mother (Wood, 1993). Trophoblast cells selectively express MHC class I antigens that are major determinants recognized by

CD8 lymphocytes to distinguish self from nonself, while expression of MHC class II recognized CD4 lymphocytes trophoblast is absent (Evahold et al., 1993). The conceptus could potentially be alloantigen to induce MHC-mediated immune response during pregnancy even though expression of polymorphic MHC class I protein on regions of trophoblast in direct contact with maternal blood remains a subject of some controversies (Lala et al., 1983; Redline and Lu, 1989). In mice, significant surface expression of paternal MHC appeared at day 13 of a 21 day pregnancy (Raghupathy et al., 1981; Hedley et al., 1989). Trophoblast cells from 13 to 16 day embryos were susceptible to lysis by cytotoxic lymphocytes (CTL) (Croy and Rossant, 1987). Murine trophoblast lining maternal blood spaces lacked MHC antigens, but other interstitial trophoblast expressed fetally derived polymorphic MHC class I determinants (Redline and Lu, 1989). The MHC class I trophoblast was most prominent in the maternal decidua basalis. This is a region rich in maternal cells called granulated metrial gland (GMG) cells that are natural killer (NK)-like cells with putative immunoregulatory actions (Redline and Lu, 1989).

In humans, invasive cytotrophoblast cells express a truncated class I antigen, human leukocyte antigen G (HLA-G), and both cell-associated and secreted HLA-G antigens are prominent in first trimester villous cytotrophoblast and are greatly reduced in third trimester cytotrophoblast (Kovats et al., 1990). HLA-G functions as a typical MHC class I $\alpha$ chain in that it associates with $\beta_2$ microglobulin and binds to CD8 (Sanders et al., 1991). However, there is no allelic variation in HLA-G,

suggesting HLA-G gene is not polymorphic in cytotrophoblast and may therefore not be recognized as foreign by the maternal immune system (Kovats et al., 1990). Expression of HLA-G may also protect the trophoblast against cytolytic effects of NK-like cells because NK cells preferentially recognize targets without expression of MHC (Ljunggren and Karre, 1990). Another possible mechnism by which maternal T cells become activated by fetal antigen involves leakage of fetal cells into the maternal circulation (Philip et al., 1982). Trophoblast cells can enter the maternal circulation and are trapped in the lungs (Hunt et al., 1984) but it is not known whether these cells are able to stimulate MHC antigen-specific immune responses (Hunt et al., 1984).

The human uterine endometrium contains large granular lymphocytes believed to function as NK cells (King and Loke, 1991). Human trophoblast is resistant to lysis by decidual large granular lymphocytes, but becomes susceptible when the decidual lymphocytes are transformed into lymphokine activated killer (LAK) cells by IL-2 (King and Loke, 1990a). In addition, exposure of trophoblast to interferon (IFN)-$\gamma$ induces expression of MHC class I molecules on trophoblast and makes trophoblast cells more susceptible to lysis by allospecific cytolytic lymphocytes (CTL) (King et al., 1987; Grabowska et al., 1990; Tian and King, 1994).

Activation of Uterine Lymphocytes During Pregnancy

The allogeneic conceptus creates the potential for maternal immune responses. In humans, there are signs of local activation of the maternal immune system in decidua of early pregnancy. Between 5 and 8 wk of gestation, the endometrium

consists of many endometrial glands and a markedly decidualized endometrial stroma. Numerous lymphoid cells are present within the decidualized stroma (Lin et al., 1991; King and Loke, 1991). Many of these lymphocytes have a large, irregular nucleus with relatively abundant cytoplasm and have been called large granular lymphocytes (LGL). These lymphocytes express a cytotoxic protein called perforin (Henkart, 1985) which play a role in lysis of target cells and is found in the cytoplasm of CTL, NK cells, LAK cells, and $\gamma\delta$ $TCR^+$ cells (Groscurth et al., 1987; Ojcius and Young, 1990; Tschopp and Nabholz, 1990). The LGL cells constitute 70-80% of leukocytes in the uterus compared to less than 1% of peripheral blood lymphocytes during pregnancy (Mincheva-Nisson et al., 1994).

Human uterine LGL are mainly composed of $CD56^+TCR$ $\gamma\delta^+$ cells, $CD56^+$ $\gamma\delta$ $TCR^-$ cells, $CD8^+$ $\alpha\beta$ $TCR^+$ cells, and $CD4^+$ $\alpha\beta$ $TCR^+$ cells (Mincheva-Nilsson et al., 1994). In addition, most intraepithelial lymphocytes (IEL) are $CD56^-$ $\gamma\delta$ $TCR^+$ lymphocytes in the human endometrium (Mincheva-Nisson et al., 1994). The CD56 antigen is expressed on NK cells in peripheral blood but decidual $CD56^+$ cells do not express another NK marker, CD16 (King et al., 1989; Ritson and Bulmer, 1987). The uterine LGL are functionally different from classical NK cells in that they exhibit lower levels of NK activity against the prototypic NK target cell line, K562 (Nagler et al., 1987). CD8 antigen is expressed on cytotoxic/suppressor T cells. Interestingly, no B cells are found in the endometrium.

In mice, decidual cells share several surface markers in common with

lymphomyloid cell surface markers such as Thy-1 and Mac-1, suggesting some decidual cells are possibly of bone marrow origin (Kearns and Lala, 1985). Decidual cells migrate to or differentiate in the uterus in response to maternal hormones or are stimulated by the conceptus or foreign implants (Zheng et al., 1991). During pregnancy, the two major subpopulations of uterine lymphocytes in the decidual tissue of uterus are NK cells and $\gamma\delta$ TCR$^+$ cells (Heyborne et al., 1992; Linnemeyer and Pollack, 1994; Susuki et al., 1995). In early pregnancy, the decidual NK cells have high levels of cytolytic activity (Gambel et al., 1985). By midgestation, NK activity is decreased and during late pregnancy, NK activity is undetectable (Gambel et al., 1985). Also during late pregnancy, granulated metrial gland (GMG) cells becomes a prominent feature of the decidua (Stewart et al., 1978). GMG cells are NK-lineage cells (Parr et al., 1990) that exhibit no NK lytic activity unless induced by IL-2 (Stewart and Mukhtar, 1988; Parr et al., 1990; Croy et al., 1991). They reach sizes in excess of 50 $\mu$m and have numerous large granules that contain perforin and serine esterase molecules, which are associated with NK lytic activity (Parr et al., 1990; Lin et al., 1991).

A specific subset of $\gamma\delta$ TCR$^+$ cells bearing a V$\gamma$6V$\delta$1 encoded TCR populates the maternal-fetal interface in mice (Heyborne et al., 1992). This subset of $\gamma\delta$ TCR$^+$ cells increased nearly 100 fold in allogeneic pregnant animals (C57B1/10 x BALB/c) compared with nonpregnant animals (C57B1/10) (Heyborne et al., 1992). About one-third of $\gamma\delta$ TCR$^+$ cells from pregnant uterus expressed IL-2 receptors (Heyborne et

al., 1992). All γδ $TCR^+$ cells are maternally-derived based on analysis of MHC antigen phenotype (Suzuki et al., 1995). This subset of uterine γδ $TCR^+$ cells is in an activated state during pregnancy compared to non-pregnant mice and seems to be more activated in allogeneic pregnancy than those in syngeneic pregnancy (Suzuki et al., 1995). For example, pregnancy increased both size and granularity of uterine γδ $TCR^+$ cells, especially in allogeneic pregnancy (Suzuki et al., 1995). The proportion of uterine γδ $TCR^+$ cells positive staining for CD69 (an activation marker for T, B and NK cells) was 85.8 % in allogeneic pregnancy, 88.4% in syngeneic pregnancy and 51% in non-pregnant mice (Suzuki et al., 1995). The proportion of uterine γδ $TCR^+$ cells staining for CD25 (IL-2Rα) was 31% in allogeneic pregnant animals, 24% in syngeneic pregnant animals and 13% in non-pregnant animals. The percentages of γδ $TCR^+$ $CD44^+$ were 17% in allogeneic pregnancy and 8.9 in syngeneic pregnancy (Suzuki et al., 1995). γδ $TCR^+$ cells reacted with murine fresh trophoblast and human trophoblast cell line (JAR) to produce lymphokines with IL-2 and IL-4 activity (Heyborne et al., 1994). This trophoblast recognition is TCR dependent because only $V\gamma1^+$ clone T cells can be stimulated by trophoblast to produce lymphokines after exposure to trophoblast (Heyborne et al., 1994).

In pigs, there is extensive infiltration of neutrophils and some eosinophils into the subepithelial regions of endometrium at 2-4 days post-mating (Bischof et al., 1995). Their presence is possibly associated with local inflammation induced by mating or with the dynamic changes in structure and function of the endometrium in

preparation of implantation (Bischof et al., 1995). The numbers of lymphocytes were decreased at 10-14 day of gestation. Numbers of $CD2^+$ lymphocytes were increased; number of $CD2^+$ cells far exceeded the sum of $CD4^+$ cells and $CD8^+$ lymphocytes indicating presence of $CD4^-$ $CD8^-$ T cells. After day 25 of pregnancy, there was an increase in NK cell activity in the uterus, suggesting NK cells accumulate in the pregnant pig endometrium (Croy et al., 1988).

Possible Functions of Uterine Lymphocytes

$CD4^+$ and $CD8^+$ T cells. The function of T cells is to recognize and react to antigen by proliferation and differentiation into either $CD8^+$ effector cells mediated by MHC class I or $CD4^+$ helper (Th) cells mediated by MHC class II. Effector functions exerted by $CD8^+$ cells include cytotoxicity to target cells and suppression of immune responses. Functions of T helper cells are mediated through production of cytokines. The functions of T cells in the endometrium of pregnancy are incompletely understood. While T cells play an important role in maintaining normal pregnancy, it is pertinent that T- and B-deficient severe combined immunodeficiency disease (SCID) mice and T-cell-deficient nude mice have successful pregnancy even without T cell function (Croy and Chapeau, 1990). There is some evidence that a major role of $CD8^+$ cells is to suppress immune responses. For example, human endometrial $CD8^+$ cells are activated by the conceptus to release a 34-kDa molecule that inhibits NK cell activity (Szekeres et al., 1990). Also, administration of antibody to $CD8^+$ dramatically increased the spontaneous abortion rate of DBA/2-mated CBA/J female

mice (Clark et al., 1989b). Large $CD8^+$ suppressor cells appear in murine endometrium in response to hormonal changes of early pregnancy and suppress generation of CTL in vitro (Clark et al., 1991a). In addition to $CD8^+$ T cells, $CD4^+$ T cells produce T cell suppressive factor (Tsif) that activates $CD8^+$ suppressive cells to modulate potential maternal immune responses during pregnancy (Ribbing et at., 1988). Injection of antibody to Tsif induced abortion in mice (Beaman and Hovesland, 1988). In contrast, there is also evidence for endometrial T cells playing a role in anti-fetal responses. $CD4^+$ Th 1 cells produce IL-2 and IFN-$\gamma$ that can convert natural killer cells and $CD8^+$ T cells into LAK cells. High levels of uterine IL-2 and IFN-$\gamma$ are correlated with fetal resorption (Lala et al., 1990).

$\gamma\delta$ $TCR^+$ cells Mouse uterine $\gamma\delta$ $TCR^+$ IEL do not respond to paternal stimulator lymphocytes in pregnancy (Suzuki et al., 1995), but trophoblast cells can induce the activation of $\gamma\delta$ $TCR^+$ cells to produce lymphokines (IL-2 or IL-4); this recognition of trophoblast by $\gamma\delta$ $TCR^+$ cells is not species specific (Heyborne et al., 1994). In addition to production of lymphokines, uterine $\gamma\delta$ $TCR^+$ IEL may also suppress the maternal antifetal immune response at the maternal-fetal interface because $\gamma\delta$ $TCR^+$ IEL produce immunosuppressive growth factors such as TGF-$\beta$ (Suzuki et al., 1995).

NK-like cells. Human LGL are abundant particularly in first-trimester decidua (Kabawat et al., 1985). These cells display strong expression of the NK cell marker CD56, but not other markers such as CD16 and CD3 (Burrow et al., 1993). Functions of LGL are still not clear. One possibility is that they limit the migration

and differentiation of highly invasive trophoblast cells through production of cytokines (King and Loke, 1991). Molecules such as CSF-1, TGF-$\beta$ and GM-CSF have been found to be products of human $CD56^+$ decidual lymphocytes (Jokhi et al., 1994) and mouse uterine NK cells (Croy et al., 1991; Athanassakis, 1993). Uterine NK-like cells may have both immunological as well as nonimmunological activities. It has been suggested that they protect the embryo against viral and bacterial infections. NK-like cells are also to prevent tumor-like invasive trophoblast cells from maternal tissues (Croy, 1990a; King and Loke, 1990b; Stewart, 1990). They produce tumor necrosis factor (TNF) that inhibits trophoblast cell DNA synthesis (Yelavarthi et al., 1991). During pregnancy, uterine NK-like cells express markers associated with priming and activation for specific functions such as killing of tumor cells or cytokine production. For example, mouse NK-like cells express cytotoxic proteins such as perforin and serine esterase (Parr et al., 1990).

Two stage-specific activation surface markers for NK cells have been used to evaluate function of uterine NK cells in mice. An early marker 3C2 for NK cells can be first detected on decidual GMG cells on day 5 of pregnancy, one half day after embryo implantation. These $3C2^+$ cells exhibit LGL morphology and NK lytic activity (Linnemeyer and Pollack 1994). However, a late marker 4H14 for GMG cells can be detected on $3C2^+$ cells from day 8 to 9 of pregnant mice (Linnemeyer and Pollack 1994). $4H14^+$ decidual cells develop the GMG cell morphology, which have no NK lytic activity against YAC-1 cells (Linnemeyer and Pollack, 1994). Thus,

decidual cells from early stages of pregnancy have cytolytic activity and may attack trophoblast cells, while cells without lytic activity from the late stages of pregnancy may secrete cytokines to support embryonic development.

Factors that contribute to NK cell differentiation are incompletely understood. Cytolytic activity of decidual cells can be induced in culture by addition of $PGE_2$ (Linnemeyer and Pollack, 1993). The injection of poly(I)poly(C), which induces production of IFN$\gamma$, triggered NK cell activation and increased embryonic degeneration (Kinsky et al., 1990). This effect can be adaptively transferred with poly(I).poly(C) treated spleen cells (Kinsky et al., 1990). Embryonic degeneration due to poly(I).poly(C) can be prevented by immunizing with cells bearing paternal alloantigens before mating (Clark et al., 1986). However, the mechanism for this effect is unclear.

Mechanisms Which Regulate Maternal Immune Responses in Mice and Humans

Several factors may contribute to the maternal tolerance toward the allogeneic fetus. One is the expression of MHC class I molecules on trophoblast to protect embryo from maternal NK cell and T cell attack. A second contribution is switching of cytokine production by $CD4^+$helper T cells from Th1 responses, which favor cytotoxicity, to Th2 responses, which favor humoral immunity. The third major contributing factor is local production of immunosuppressive molecules including cytokines, steroid hormones and various endometrial or trophoblast proteins that down-regulate alloreactivity of maternal immune responses.

MHC Expression on the Trophoblast and Immune Responses

The MHC is a region of highly polymorphic genes whose products are expressed on the surface of a variety of cells. There are two different types of MHC gene products, called MHC class I and class II molecules. The function of MHC proteins are to present processed foreign antigen to T cells. MHC class I proteins are involved in presentation for CD8 cytotoxic/suppressor cells while MHC class II proteins are presented for CD4 helper T cells (Townsend and Bodmer, 1989). In tissue grafts, the polymorphic MHC expression on graft tissue is recognized as foreign antigen by host lymphocytes. Graft rejection is mediated by MHC class I-restricted $CD8^+$ T cell, which usually function as CTL (Bjorkman et al., 1987).

The conceptus is considered as an allograft because fetal cells express paternal MHC class I and II antigens and fetal tissues elicit a graft rejection response (Croy and Rossant, 1987). However, there is good evidence that the antigenicity of trophoblast is reduced because MHC antigen expression is minimized or of a variant type. For example, murine trophoblast does not express normal MHC class I or class II gene products (Sutton et al., 1983; Hunt et al., 1988) and trophoblast cells did not stimulate mixed lymphocyte reactions in mice (Hunt et al., 1985). In humans, trophoblast cells express HLA-G, a truncated MHC class I (Kovats et al., 1990), It has been proposed that the absence of MHC expression on trophoblast in mice contributes to preventing maternal anti-placenta immune responses (Wood, 1994). However, other data do not support this hypothesis in mice. It has been found that

mouse trophoblast cells express MHC class I antigen (Redman and Lu, 1989; Shorter et al., 1993). Expression of MHC class I antigens is increased by interferon $\alpha/\beta$ of trophoblast origin (Mattsson et al., 1993) and by IFN-$\gamma$ (Hager et al., 1994). Enhanced expression of MHC class I antigens by interferons may be an important mechanism to increase the efficiency of local and viral antigen presentation as well as T cell cytotoxicity (Hager et al., 1994). In horses, invasive trophoblast cells of the chorionic girdle express high levels of paternally inherited MHC class I antigens prior to migration into the endometrium to form the endometrial cups. Both maternal and paternal MHC class I antigens are expressed by the equine invasive trophoblast cells (Donaldson et al., 1994).

The expression of MHC molecules may eliminate the possibility of trophoblast lysis by uterine NK cells. The "missing self" hypothesis proposes that NK cells recognize and eliminate cells that fail to express self MHC class I molecules on the cell surface (Ljunggren and Karre, 1990). Murine and human NK cells express receptors for polymorphic MHC class I molecules that inhibit killing of target cells bearing appropriate alleles. These receptors, designated as killer-cell inhibitory receptors (KIRs) are members of the immunoglobulin superfamily (Malnati et al., 1995; Wagtmann et al., 1995; Colonna et al., 1995). Following this hypothesis, trophoblast cells not expressing MHC class I antigens would be eliminated by uterine NK cells. Thus, placental expression of MHC class I antigens may be necessary to thwart uterine NK killing. Expression of placental MHC class I antigens and its

interaction with KIRS may explain why trophoblast cells are resistant to lysis by NK cells in human and mice (King and Loke, 1991; McMaster, 1995).

Expression of MHC class I antigens on trophoblast means maternal T cells should recognize trophoblast. There is recent evidence from mice that this recognition leads to specific suppression. Pregnant mice with allogeneic pregnancy accept more tumor grafts than do syngeneic pregnant mice, and T cell phenotype and responsiveness are restored after delivery (Taturi et al., 1995). This indicates that tolerance to fetal MHC develops during pregnancy, but it is a transient process and does not result in anergy (Tafuri et al., 1995). T cells also have inhibitory MHC receptors similar to NK cells for MHC class I antigens (Lanier and Phillips, 1996). The presence of inhibitory MHC receptors on a T cells may provide a new conceptual framework for understanding the regulation of the maternal immune response to the conceptus. Taken together, data indicate that the expression of MHC class I molecules on trophoblast provide maternal NK and T cells with inhibitory signals that protect the conceptus from attack by the maternal immune system in mice and other species in which MHC class I molecules are expressed on trophoblast cells.

The Immunotrophism Hypothesis

There are two lines of evidence that immunological recognition of the fetus by the mother enhances fetal survival. First, the so-called DBA/CBA model has been used to study maternal immune responses to the conceptus. CBA/J ($H\text{-}2^{+}$) females are susceptible to increased abortion if mated to DBA/2 males ($H\text{-}2^{+}$), while mating

CBA/J females to other H-$2^d$ males does not lead to a similar rate of abortion (Croy et al., 1982). Abortion begins by about day 8.5 to 9.5, and most of the implantation sites in which will be lost are necrotic by day 13.5 to 14.5 (Croy et al., 1982). In the CBA x DBA/2 system, there is infiltration by cytotoxic lymphocytes into the decidual tissues (Clark et al., 1986). Immunization to paternal H-$2^d$ antigens protected against abortion (Chaouat et al., 1983; Kiger et al., 1985; Clark et al., 1987). Removal of T cells from pregnant mice by administration of antibodies to CD4 and CD8 resulted in reduced placental size and in some cases increased fetal resorption (Athanassakis et al., 1987) and T-cell depletion enhanced abortion rates in DBA x CBA/2J mating and blocked the protective effect of immunization (Chaouat et al., 1988). A clinical trial indicates that immunization with paternal leukocytes or third-party leukocytes or administration of antibody to paternal leukocytes can prevent abortion in couples with partner-specific losses and boosted efficacy to 70% with immunization vs. 32% or control in humans (Clark and Daya, 1991). Thus, acquisition of specific immunity could improve trophoblast functions and enhance fetal development and growth.

Immunization with paternal MHC class I antigens may support fetal development and survival as a result of cytokine production by maternal T cells. Wegmann (1987) first proposed this hypothesis and suggested that cytokines such as IL-3 and granulocyte colony-stimulating factor (GM-CSF) enhance trophoblast development and function. This hypothesis was tested by injecting small amounts of recombinant GM-CSF and IL-3 in the early stages of pregnancy in mice. Injection

of IL-3 increased fetal survival when injected into CBA/J females carrying abortion-prone CBA/J x DBA/2 fetuses from 22% to 55% (IL-3), while GM-CSF increased survival from 8% to 47%. Cytokines also increased fetal and placental weight and size, and expanded the spongiotrophoblast zone in the placenta (Chaouat et al., 1990). Increases in fetal survival as well as in placental and fetal size are also seen when mice were immunized against paternal H-$2^d$ molecule (Kiger et al., 1985). Anti-CD8 antibody completely abrogated the ability of GM-CSF to suppress anti-trophoblast killer cell activity (Clark et al., 1994). This suggests that GM-CSF acts indirectly to prevent abortion in DBA/2-mated CBA/J mice through a mechanism that requires $CD8^+$ maternal T cells, and that systemic regulation of the level of anti-trophoblast killer cell activity may determine the success or failure of pregnancy in this system (Clark et al., 1994). GM-CSF administration also rapidly down-regulated natural effector cells capable of killing a trophoblast target cell line in vitro. Interleukin-2, TNF-$\alpha$ and IFN-$\gamma$ have the opposite effect to IL-3 and GM-CSF since these cytokines increase fetal resorption rates in abortion-prone (CBA/J x DBA/2) and non-abortion prone (CBA/J x BALB/c, C3H x DBA/2) matings (Chaouat et al., 1990). For example, 1000 units of TNF-$\alpha$ increased resorption rate from 43 to 79% in CBA x DBA/2 mating, from 7 to 89% in CBA x BALB/c mating and from 5 to 47% in C3H x DBA/2 mating (Chaouat et al., 1990). Interferon-$\gamma$ and IL-2 enhanced resorption from 38 to 68% and 76% respectively in the CBA/J x DBA/2 mating combination, whereas resorption rates in CBA/J x BALB/c matings were enhanced

from 6 to 44% and 55% (Chaouat et al., 1990).

GM-CSF is a hemopoietic cytokine that has effects on the proliferation, differentiation and function of placental cells and growth (Athanassaki et al., 1987; Pollard, 1990; Garcia-Lloret et al., 1994). The cytokine may improve embryonic survival by reversing deleterious effects of IFN and TNF on the development of the conceptus in CBA x DBA system. It has been reported that GM-CSF not only down-regulate IL-2 receptor expression (Hancock et al., 1988) and reduces TNF-$\alpha$ production (Vermeulen et al., 1987) but also activates natural suppressor cells to produce TGF-$\beta$ (Moore et al., 1990). In fact, GM-CSF causes a modest increase in [$^3$H] thymidine incorporation by human trophoblast cells in culture (Loke et al., 1992). Recently, this finding for both GM-CSF and CSF-1 has been confirmed in murine trophoblast cells (Athanassakis, 1993). In contrast, IL-3 had only a mild stimulatory effect, and IL-2 did not alter the level of DNA synthesis in these cells (Drake and Head, 1994). In addition, GM-CSF is known to enhance the function and increase the survival of mature cells by preventing apoptosis and increase human first trimester trophoblast cell numbers in vitro (Colotta et al., 1992). GM-CSF also caused a threefold increase in human placental lactogen secretion from trophoblast cells at day 2 to day 5 of culture (Garcia-Lloret et al., 1994).

The immuntrophism hypothesis has been challenged by several lines of evidence. First, GM-CSF can be produced by epithelial cells, fibroblasts and macrophages in addition to lymphocytes (Munker et al., 1986; Pollard et al., 1987;

Garcia et al 1994). Neither decidual supernatants from days 5.5-6.5 pregnant mice having high abortion rates (CBA/J-mated DBA/2) nor from low-abortion-rate (C3H/HeJ mated DBA/2) had effects on trophoblast development, suggesting that GM-CSF is not crucial for success of pregnancy (Lea and Clark, 1993). Second, *scid/scid.bg/bg* mice deficient in NK cells and lacking functional T and B cells have normal litter size, low rates of abortion and normal levels of placental growth stimulating factors released from decidua (Clark et al., 1989; Croy and Chapeau, 1990). Third, treatment of DBA x CBA pregnant mice with antibody to asialo-GM1, which is the surface marker for murine uterine NK linkage cells, is able to prevent abortion, suggesting activated uterine lymphocytes contribute to rejecting the conceptus (Clark et al., 1991).

Redirection of Cytokine Production from Helper T cells During Pregnancy

T-helper cells can be defined based on the cytokines they produce. Th1 cells promote cellular immunity and produce IL-2, IFN-$\gamma$ and TNF, while Th2 cells, which are involved in B cell development and antibody production, produce IL-3, IL-4, IL-5, IL-10, TGF-$\beta$ and GM-CSF (Mosmann et al., 1986). The cytokines produced by Th2 cells enhance Th2 development and inhibit Th1 development, while Th1 cytokines stimulate development of Th1 and inhibit development of Th2 (Paul and Seder, 1994). Wegmann (1993) proposed a hypothetical concept that the fetal-placental unit redirects $CD4^+$ T helpers to develop into Th2 cells while development of Th1 cells is inhibited. In mice, the fetoplacental unit is able to stimulate uterine lymphocytes to produce

more IL-4 (1658 U vs 184 U), IL-5 (1.2 ng vs $<0.3$ ng) and IL-10 (30 U vs 1.2 U) than Con A-stimulated spleen cells, but less IFN-$\gamma$ (2.7 U vs 66 U) (Wegmann et al., 1993). T cell depletion experiments showed that biologically active GM-CSF and IL-3 are produced by uterine T cells at the 11th day of pregnancy (Athanassakis et al., 1993), and GM-CSF augments the immunosuppressive capacity of murine lymphocytes (Morrissey and Ireland, 1991). Interleukin-2, TNF-$\alpha$ and IFN-$\gamma$ have deleterious effects on the trophoblast (Wegmann et al., 1993). Incubation with TNF-$\alpha$ alone induced a loss of trophoblast viability and reduced DNA content in mice (Yui et al., 1994). The combination of TNF-$\alpha$ and IFN-$\gamma$ enhanced the deleterious effect and increased the apoptosis of trophoblast (Yui et al., 1994). IFN-$\gamma$ activates NK cells and inhibits the secretion of GM-CSF (Robertson et al, 1992), and induces more expression of MHC class I antigen so that trophoblast cells are more susceptible to lysis by NK cells (Ljunggren and Karre, 1990).

Clinical observations in humans support the Th1/Th2 switch hypothesis (Hill et al., 1995). Supernatants from 160 of 244 trophoblast-activated peripheral blood mononuclear cells from women with unexplained recurrent abortion (URA) had embryotoxic activity while supernatants from normal pregnant women did not. IFN-$\gamma$ was detected in supernatants from 125 of 244 URA patients and TNF-$\alpha$ and TNF-$\beta$ were detected from 160 of 244 URA patients. However, none of the supernatants from controls with normal pregnancy were positive for TNF-$\alpha$ and $\beta$. In contrast, trophoblast-activated lymphocyte supernatants from reproductively normal women and

men were not embryotoxic and did not contain Th1-type cytokines, but most contained the Th2-type cytokine IL-10. Three supernatants from reproductively normal women also contained IL-4. Therefore, Th1-type immunity to trophoblast is associated with URA and may play a role in reproductive failure. It follows that Th2-type immunity may be a natural response to trophoblast contributing to successful pregnancy.

The mechanism which triggers the initial production of Th2 cytokines during pregnancy is unknown. Generally, activation of T cells can be induced by antigen-presenting cells and costimulatory proteins on the antigen-presenting cells. Recently, it was found that the most potent costimulatory proteins belong to a family called B7. Two of these, B7-1 and B7-2, seem to influence Th1/Th2 ratios (Kuchroo et al., 1995). The relative amounts of B7-1 and B7-2 determine whether the newly activated T helper cells will produce Th1- or Th2-type cytokines, which in turn influence further differentiation to Th1 or Th2 cells (Freeman et al., 1995).

According to Wegmann's hypothesis, IL-10 derived from maternal decidua may play a critical role in shifting T helper cytokine responses from Th1 to Th2. Redirection of cytokines produced by T helper cells may be regulated by pregnancy hormones rather than by costimulation molecules on antigen-presenting cells. Progesterone may be involved in Th1/Th2 switching because culture of human lymphocytes with progesterone increased IL-4 secretion and expression of the Th2 marker CD30 (Piccinni et al., 1995).

The Role of Suppressor T Cell-Mediated Suppression

Suppressor T cells comprise a class of lymphocytes distinct from T helper and cytolytic T lymphocytes. Their function is to inhibit competent antigen-specific T and B lymphocytes. Suppressor T cells may be critical for preventing immune responses to self antigens that do not induce tolerance because they are not accessible to immature lymphocytes. During pregnancy, a novel $CD8^+$ population of small lymphocytes with suppressive activity accumulates in the uterine decidua of humans and mice (Slapsys et al., 1988). In mice, $CD8^+$ suppressor cells have been isolated from the uterine lining after mating which are progesterone receptor-positive and can be stimulated by progesterone to release a soluble molecule of approximately 34 kDa (Brierley and Clark, 1987). This molecule inhibits activity of NK cells and generation of lymphokine-activated killer cells (Szekeres et al., 1985) and prevents abortion in mice (Szekeres and Chaouat, 1990). Deficiency of progesterone receptor-positive cells is associated with abortion in humans (Szekeres et al., 1989). T suppressor inducing factor (Tsif), which is produced by $CD4^+$ cells, can be found in murine uterus and activates $CD8^+$ suppressor cells (Ribbing et al., 1988).

Placental-Associated Suppressor Factors

Several factors from placenta have immunosuppressive activity. A suppressor factor from human trophoblast-derived choriocarcinoma cell line has been identified as a low molecular-weight compound in the range of 5-6 kDa, composed predominantly of hydrophilic amino acids (Krishnan et al., 1995). The factor mediated a dose-dependent suppression of proliferative responses of both human and

murine T lymphocytes in vivo and in vitro, but did not affect other lymphoma cells or B cells (Krishnan et al., 1995). In addition, the factor completely suppressed the IL-2-independent proliferative responses of T cells by PMA but failed to suppress antigenic expression of activation-associated CD 25 molecules (Matsuzaki et al., 1991).

Recently, a mouse placental immunoregulatory factor has been identified (Bleux et al., 1995). This placental factor is 40 kDa glycoprotein extracted from mouse placenta that not only suppressed T and B lymphocyte proliferation, cytotoxic T lymphocyte generation, lymphokine-activated killer cell development, as well as natural killer cell activity, but also highly inhibited secondary antibody responses as well as cellular responses such as local graft-versus-host reactions (Bleux et al., 1995).

Human pregnancy zone protein (PZP) Another suppressive protein of placental origin, pregnancy zone protein (PZP), is quantitatively the most important pregnancy-associated protein synthesized by human trophoblast and is highly homologous to $\alpha_2$-macroglobulin (Sottrup et al., 1984). Pregnancy zone protein is able to bind proteinases which cleave internal thiol esters (Jensen et al., 1993). Receptors for PZP have been found in the human placental syncytiotrophoblast (Jensen et al., 1988). Pregnancy zone protein is a potential immunoregulatory protein during pregnancy because it inhibits production of IL-2 by activated T cells but does not affect IL-2 receptor expression on T cell activation (Saito et al., 1990).

Pregnancy zone protein suppressed DNA synthesis following stimulation with PHA, Con A or CD3 antigen and this effect of PZP was mediated by a reduction in IL-2 production (Saito et al., 1990). Actions of PZP were abolished by exogenous recombinant IL-2 administration but not affacted IL-2R expression (Saito et al., 1990). Also, PZP did not affect the proliferation of T cells following stimulation with the combination of the calcium ionophore, A23187, and PMA (Saito et al., 1990). Pregnancy zone protein also abrogated purified protein derivative (PPD)-induced leukocyte migration in skin and caused a dose dependent inhibition of lymphocyte transformation after stimulation with PHA, PPD and MLR (Birkeland et al., 1979).

α-Fetoprotein (AFP) AFP is the dominant serum glycoprotein of fetal life and present in amniotic fluid but is expressed only in low nanogram levels in adult sera (Hoskin et al., 1985). A number of diverse biologic functions have been attributed to AFP, including osmotic and carrier functions and immunosuppressive activity (Hoskin et al.,1985). Proliferation of $CD8^+$ T cells induced by PHA, Con A and MLR was susceptible to inhibition by AFP in humans (Hooper and Evans, 1989) and mice (Hoskin and Murgita, 1989; Rueda et al., 1990).

Trophoblast-Derived Cytokines and Hormones Human trophoblast cells produce several cytokines such as IFN (Zdravkovic et al., 1994), IL-4 and CSF-1 (Haynes et al., 1993) and IL-1 (Librach et al., 1994). However, the role of trophoblast-derived cytokines in regulating maternal immune responses is still not clear. For example, human placental trophoblast cells produce IFN-$\beta$, which can be produced

predominantly by trophoblast when simulated with viruses (Zdravkovic et al., 1994). Trophoblast-IFN-$\beta$ suppresses mitogen-induced lymphocyte proliferation at concentrations of 10-1000 IU/ml. Thus, it is possible that human trophoblast-IFN-$\beta$ may participate in the local control of maternal immune responses during pregnancy at the feto-maternal interface.

Human trophoblast hormones may also be involved in regulating the immune responses during pregnancy. For example, human chorionic gonadotropin and placental lactogen inhibit IL-2 secretion from lymphocytes while increasing IL-1$\beta$, IL-6 and INF-$\alpha$ (Schafer et al., 1992). In addition, trophoblast cells contribute to local high concentrations of progesterone during late pregnancy in some species. Progesterone increased numbers of $CD8^+$ suppressor cells in the gravid uterus, inhibited NK cells (Szekeres et al., 1985) and LAK generation (Szekeres et al., 1988), and caused redirection of cytokine production by peripheral blood human T cells (Piccinni et al., 1995). Regulation of local immune responses by progesterone during pregnancy in sheep will be discussed later.

The Uterine Immunosuppressive Factors

The endometrial mucosa is unique amongst mucosal sites in that it is exposed to both infectious agents and the allogeneic fetus. During pregnancy, a large number of secretory components are produced by the endometrium. These substances include enzymes, their inhibitors, hormones, transport proteins and bioactive peptides with diverse functions. There are several molecules implicated in regulation of immune

function among them.

Placental ferritin (PLF). Placental ferritin is a tumour associated protein composed of a 43-kDa subunit (p43) and ferritin light chains that causes suppression of PHA-induced blastogenesis at concentrations as low as 0.25 $\mu$g/ml (Matzner et al., 1985). The p43 subunit is produced by activated uterine $CD4^+$ cells while a subset of $CD8^+$ cells has receptors for p43 (Garty et al., 1995). Immunosuppression by PLF may play an important role in the down-regulation of the human immune system during pregnancy. Alloimmunization activates immunosuppression by PLF in the human (Moroz et al., 1993). Vaccination of women with paternal lymphocytes resulted in immunosuppression due to PLF (Moroz et al., 1993). The highest immunosuppressive effect was reached after three vaccinations, and the increase in PLF immunosuppressive effect was accompanied by the appearance of cytotoxic antibodies in serum directed against leukocyte antigen (Moroz et al., 1993).

Placental protein 14 (PP14). This is the major secretory protein in the human endometrium during the mid- to late-luteal phase of the menstrual cycle and early first trimester pregnancy (Rutanen et al., 1985; Bell and Dore, 1987; Julkunen et al., 1988). The protein has a molecular weight of 28 kDa. PP14 is immunologically identical to progesterone-dependent endometrial protein, also called pregnancy-associated endometrial $\alpha_2$-macroglobulin or chorionic $\alpha_2$-globulin (Seppala et al., 1988). Although PP14 is detected in the endometrial secretion and the peripheral blood of nonpregnant women, the production of these proteins increases progressively

after decidualization, and both protein levels remain high throughout pregnancy, especially at the fetomaternal interface (Julkunen et al., 1985; Rutanen et al., 1987).

PP14 suppressed lymphocyte IL-2 production induced by PHA and inhibited the release of soluble CD25 (IL-2R) into the culture supernatants (Pockley and Bolton, 1989). PP14 inhibited the production of IL-1 from mitogen-stimulated PBL cultures. Inhibition was effective on both PHA and lipopolysaccharide(LPS)-induced IL-1 secretion. PP14 also inhibited incorporation of [$^3$H]thymidine into PHA-stimulated lymphocytes and inhibition was partially reversed by the addition of exogenous IL-1 to the cultures (Pockley and Bolton, 1990). Also, treatment of purified LGL with 5-50 $\mu$g/ml PP14 resulted in suppression of cytotoxicity against K562 target cells in a 4 h $^{51}$Cr release assay (Okamoto et al., 1991). These results indicate PP14 is another candidate for suppression of the function of uterine NK cell in humans.

Transforming growth factor (TGF)-$\beta$. TGF-$\beta$ belongs to a superfamily of structurally related regulatory proteins. The most abundant isoform is a 25 kDa homodimer. TGF-$\beta$ has regulatory effects on a broad spectrum of cell types. Because TGF-$\beta$ possesses both pro-inflammatory and anti-inflammatory activities, it plays a pivotal role in the immune system (Wahl, 1991; 1992). This molecule is an immunosuppressive cytokine which inhibits T and B cell proliferation (Kehrl et al., 1986) as well as macrophage maturation (Strassmann et al., 1988) and activation (Kehrl et al., 1986). Also, TGF-$\beta$ inhibits activity of NK cells and LAK cells (Rook et al., 1986; Espevik et al., 1987) and blocks cytokine production by T cells (Espevik

et al., 1988).

TGF-$\beta$ has been found to be a product of natural suppressor cells present in the uterus at day 4 to 5 after implantation in the mouse (Clark, 1988). In the human, both $CD56^+$ as well as the $CD56^-$ uterine cells could release biologically active TGF-$\beta$ in culture (Clark, 1994). The uterine TGF-$\beta$, which has a lower molecular mass (20-23 kDa) than TGF-$\beta_2$ (25 kDa), has been shown to be released by non-T non-B suppressor cells of murine decidua (Clark et al., 1995). Activated endometrial $\gamma\delta$ $TCR^+$ cells from mouse endometrium released biologically active TGF-$\beta$ in vitro (Suzuki, 1995). The neutralization of immunosuppressive activity by passive immunization against TGF-$\beta$ boosted the abortion rate (Clark, 1991). Furthermore, antibody against pregnancy-associated natural suppressor cells can produce abortion (Hoskin et al., 1989). Taken together, results suggest that TGF-$\beta$ is produced by $CD56^+$ and $\gamma\delta$ $TCR^+$ cells to suppress other endometrial lymphocytes including lymphokine activated lymphocytes and natural killer cells (Suzuki et al., 1995). In the human, the IL-2-induced activation of lysis of sorted decidual CD16- $CD56^{bright}$ NK cells cannot be suppressed by TGF-$\beta_2$ (Saito et al., 1993).

Prostaglandin $E_2$. Prostaglandin (PG) $E_2$ is produced by both fetal placenta and maternal endometrium (Parhar and Lala, 1986; Mattews and Searl, 1987). The secretion of $PGE_2$ is enhanced by progesterone (Yagel et al., 1987). Prostaglandin $E_2$ exerts inhibitory effects on PHA-induced lymphocyte proliferation and MLR at final concentrations of $10^{-8}$ M or higher (Low and Hansen, 1988; Reed and DeGowin,

1992; Roth et al., 1992). Effects of $PGE_2$ on lymphocyte function can be reversed by a prostaglandin inhibitor, indomethacin (Lala, 1988). Prostaglandin $E_2$ suppressed lymphocyte function by inhibiting IL-2 receptor generation and IL-2 production (Parhar et al., 1989). Effects of $PGE_2$ are through activation of adenylate cyclase, which converts ATP into 3',5'-cyclic AMP leadin to activation of protein kinase A (PKA). Activated PKA regulates cAMP response element binding proteins to inhibit lymphocyte proliferation (Bauman et al., 1994).

Data from experiments to test effects of inhibitors of $PGE_2$ synthesis on abortion in mice have not yielded consistent results. Treatment of CD1 mice with indomethacin caused abortion rates of up to 100% (Lala et al., 1990) but there was no effect of indomethacin on pregnancy rate of C3H, CBA/J mice (Clark, 1991). The effect does not necessarily imply that pregnancy was disrupted because of alteration in maternal immune function becuase $PGE_2$ is also involved in implantation and early decidualization (Lala et al., 1990).

High molecular weight uterine macromolecules. Murine macromolecules ($\geq$ 300 kDa and 60-100 kDa) released from non-T cells of pregnant decidua apparently serve as binding or carrier molecules for proteins such as TGF-$\beta$ which are inhibitors of lymphocyte activation (Clark et al., 1988). In pigs, uterine fluid collected at day 15 of the estrous cycle and pregnancy contained a $\geq$ 230 kDa component which suppressed thymidine incorporation into PHA-induced lymphocytes (Segerson et al., 1981). Antibody to TGF-$\beta$ reduced the suppressor activity of this macromolecule by

34.2% (Segerson, 1995).

Interactions Between the Maternal Immune System and the Conceptus in Sheep

Like other ruminants, the sheep placenta is of an epitheliochorial type (Wooding, 1982). Chorionic villi from cotyledonary extraembryonic membranes penetrate into button-like projections of maternal endometrium. The resultant structures form chorionic cotyledons and maternal caruncles are refered to as placentomes. Trophoblast invasion of maternal tissue which occurs only in the placentomes and is limited primarily to a population of fetal chorionic binucleate cells (BNC) which migrate through the chorionic tight junction to fuse with uterine epithelial cells or their derivatives. As a result, a fetomaternal syncytium forms at the fetomaternal interface (Wooding, 1992). The binucleated cells are directly involved in the modification of the uterine epithelium to produce and deliver protein and steroid hormones (Wooding, 1992), beginning at implantation and continuing until term (Wooding, 1992). The anatomical features of the interactions between maternal tissue and the conceptus cause major differences in the maternal immune responses to the conceptus in the placentomes and interplacentomal regions of pregnant uterus (Gogolin-Ewens et al., 1989).

MHC Expression in the Placental Tissues

Using monoclonal antibody to MHC class I molecules (Gogolin-Ewens et al., 1989), expression of MHC class I molecules was detected on interplacentomal uterine epithelial cells and on placentomal epithelial cells prior to the formation of syncytium

(i.e., day 16-18 of gestation). However, MHC class I proteins are not found on fetal placental tissue cells (Gogolin-Ewens et al., 1989). MHC class II molecules are not detected in the fetal portion of plancentomes throughout pregnancy (Gogolin-Ewens et al., 1989). Despite reduced antigenicity of the placenta in sheep, antibody to fetal leukocytes was detected in maternal blood (Ford and Elves, 1974; Stear and Sponnner, 1983). The lack of expression of MHC class I molecules on the trophoblast may make placental trophoblast a target for uterine NK cells because target cells that completely lacks MHC molecules are susceptible to NK cell lysis (Lanier and Phillips, 1996).

Uterine Intraepithelial Lymphocytes During the Estrous Cycle and Pregnancy

There are three distinct subpopulations of uterine IEL. A population of $CD8^+$ $CD45R^-$ $\gamma\delta$ $TCR^-$ cells comprises about 50% of IEL during the estrous cycle, and the remainder of IEL are comprised of equal numbers of $CD8^+$ $CD45R^+$ $\gamma\delta$ $TCR^+$ cells and $CD8^+$ $CD45R^+$ $\gamma\delta$ $TCR^-$ cells (Meeusen et al., 1993). So far, no $CD4^+$ helper T cells, B cells and NK cells have been found in IEL during cycle and pregnancy (Gogolin-Ewens et al., 1989). There are several changes in uterine IEL during pregnancy. First, there are fewer T lymphocytes in the placentomal tissues compared to the interplacental regions (Gogolin-Ewens et al., 1989). This suggests the maternal response to the presence of the conceptus is different in the interplacentomal regions from the placentomes. The distribution of lymphocytes may be associated with MHC expression on placenta and uterine epithelium. Secondly, there is an increase in

number of granulated $CD8^+$ $CD45R^+$ $\gamma\delta$ $TCR^+$ IEL in the uterine epithelium in late pregnancy (Meeusen et al., 1993). During late pregnancy, the percentage of IEL is increased to 10% of all cells in the uterine epithelium compared to approximately 1% in the uterine epithelium from non-pregnant and early pregnant uterus (Meeusen et al., 1993). This granulated IEL population has been identified as the $CD8^+CD45R^+\gamma\delta$ $TCR^+$ population of lymphocytes. Third, evidence indicates that the $CD8^+CD45R^+$ $\gamma\delta$ $TCR^+$ IEL become activated during late pregnancy. These cells have exceptionally large cytoplasmic granules (Meeusen et al., 1993).

One possible function of uterine IEL is to discriminate self from non-self antigens in responses to microbial or viral infection. Both the $CD8^+$ $CD45R^-\gamma\delta$ $TCR^-$ and $CD8^+$ $CD45R^+\gamma\delta$ $TCR^-$ populations are presumablly $\alpha\beta$ $TCR^+$ and may function as MHC I class-restricted $CD8^+$ cytolytic cells to lyse target cells. They could also respond to trophoblast by transforming lymphocytes into LAK cells or uterine suppressor cells as found in the mouse (Szekeres- et al., 1985; Croy, 1990a), and the human (King and Loke, 1991). The $CD8^+$ $CD45R^+\gamma\delta$ $TCR^+$ may be not MHC-restricted and could function as natural killer-like cells. This population of cells is greatly increased in numbers and has high degree of granularity during late pregnancy (Meeusen et al., 1993). This population of cells may play a significant role in regulating other population lymphocytes, because murine uterine $\gamma\delta$ $TCR^+$ IEL produce TGF-$\beta$ to suppress proliferation of other populations of lymphocytes (Suzuki et al., 1995). Another possibility is this population of IEL may produce cytokines to

enhance embryonic development during pregnancy like murine uterine $\gamma\delta$ $TCR^+$ IEL (Heyborne et al, 1994) or human LGL cells (King and Loke, 1991).

Uterine Immunosuppressive Factors

Similar to humans and mice, there are a variety of secretory products of the trophoblast and endometrial cells in the uterus during pregnancy in sheep. Some of the following immunoregulatory substances may participate in modulating maternal responses to the conceptus at different stages of pregnancy.

Progesterone Progesterone is essential throughout pregnancy to maintain a proper uterine environment for maintaining pregnancy. One function involves induction of several endometrial proteins (Hansen et al., 1987; Moffatt et al., 1987; Vallet et al., 1991). Progesterone is derived predominantly from the corpus luteum before day 50 of pregnancy and the placenta thereafter (Heap and Flint, 1984). A variety of studies have supported the notion that progesterone exerts important influences on immunological functions in the uterus. The first observation was made by Black et al. (1953) who found that progesterone enhanced the development of pyometra in inoculated rabbits. Later, it was found treatment with progesterone enhanced survival of xenogeneic tumor cells implanted in hamster uteri; rejection occurred promptly after cessation of progesterone treatment (Moriyama and Sugawa,1972). Administration of progesterone also increased survival of intrauterine skin grafts in ovariectomized sheep uteri (Hansen et al., 1986).

Long-term (60 days) treatment of ovariectomized ewes results in a decline in

numbers of $CD45R^+$ lymphocytes and MHC class $II^+$ cells in the endometrium (Gottshall and Hansen, 1992). These data suggest progesterone regulates lymphocyte population and/or trafficking and also reduce expression of MHC class II antigen on uterine cells. Progesterone can inhibit mitogen-induced peripheral blood lymphocyte proliferation directly at concentrations of $10^{-6}$ and $10^{-5}$ mol/l (Low and Hansen, 1988; Monterroso and Hansen, 1993). These concentrations may be found in the sheep at the maternal-fetal interface during mid and late pregnancy.

A variety of studies have been performed to study the mechanisms by which progesterone regulates lymphocyte functions. Direct actions of progesterone on lymphocyte proliferation are not mediated through interaction with the progesterone receptor because the steroid receptor antagonists, RU-3044 and RU-486, did not block inhibitory effects of progesterone on lymphocyte proliferation (Monterroso and Hansen, 1993). Moreover, effects of progesterone on uterine immune function, i.e., skin graft rejection (Hansen et al., 1986) and lymphocyte numbers (Gottshall and Hansen, 1992) occur at progesterone concentrations too low to directly inhibit lymphocyte function. It has been proposed by Hansen and Liu (1994) that progesterone indirectly regulates lymphocyte function by inducing a pair of endometrial serpin-like proteins, which are called uterine milk proteins (UTM-proteins) and which have immunosuppressive activity (see UTM-protein section in this review).

IFN-$\tau$ Interferon-$\tau$ also called ovine trophoblast protein-1, is a 20-kDa embryonic

secretory product from day 13-21 of pregnancy (Bazer, 1989). Amino acid sequencing as well as molecular cloning first revealed the relationship between this protein and IFN-$\alpha_2$ (Imakawa et al., 1987). A related protein bTP-1 or bovine IFN-$\tau$ is produced by the bovine conceptus (Helmer et al., 1987). IFN-$\tau$ displays classical interferon-like biological activities including antiviral (Pontzer et al., 1988) and antiproliferative activities (Pontzer et al., 1991). Nevertheless, the major physiological role of IFN-$\tau$ is to block regression of the corpus luteum and allow for establishment of pregnancy (Roberts et al., 1992). Like other interferons, IFN-$\tau$ possesses immunosuppressive activities and inhibits mitogen-induced proliferation of sheep PBL (Newton et al.,1989), mouse spleen cells and human PBL (Assal et al., 1993). Both human CD4 and CD8 T cell subsets were inhibited by IFN-$\tau$ in a PHA-induced proliferation assay (Assal et al., 1993). However, IFN-$\tau$ enhances lytic activity of natural killer-cells towards K562 target cells in vitro (Tuo et al., 1993).

The role of IFN-$\tau$ in regulation of uterine lymphocyte function during pregnancy is unknown. The antiproliferative effect of IFN-$\tau$ on lymphocyte proliferation may indicate that it decreases maternal immune responses to the conceptus. There is a decline in uterine IEL at day 20 of pregnancy (Staples et al., 1983). Conversely, it is possible that IFN-$\tau$ causes activation of NK-like cells. Such activated NK cells could produce GM-CSF (Hancock et al., 1988), which increases embryonic secretion of IFN-$\tau$ (Imakawa et al., 1993). However, uterine NK cells have not been identified yet in sheep. Any action of IFN-$\tau$ is likely to be limited in

time because embryonic secretion is restricted to day 13-21 of pregnancy (Bazer, 1989).

Placental Tissue-Derived High Molecular Proteins Cultured placentomal cotyledons from mid and late gestation released two non-dialyzable macromolecules which inhibit lymphocyte proliferation induced by PHA, PWM and MLR but not by Con-A (Low et al., 1991). One fraction had a molecular weight of 4 $x10^6$ Da and another had a molecular weight of 4.6-16.2 x $10^4$ estimated by gel filtration chromatography (Low et al., 1991). The inhibitory activity of the high molecular weight fraction was destroyed by sodium peridate oxidation whereas the low molecular weight fraction was sensitive to pronase. Since it is not known whether both molecules also inhibit non-lymphoid cell lines, these molecules may have non-specific inhibitory effects on lymphocyte proliferation.

Uterine Milk Proteins Are Immunoregulatory Proteins

One of the mechanisms by which progesterone regulates lymphocyte functions could be through induction of proteins with immunosuppressive activity. Under the influence of progesterone, uterine endometrium produces a pair of structurally related basic glycoproteins of $M_r$ = 55,000 and 57,000 that are called uterine milk proteins (UTM-proteins) (Moffatt et al., 1987b) or uterine pregnancy protein (Zhang et al., 1989). UTM-proteins are major products in uterine secretions after day 20 of pregnancy and can be induced by as early as 10 days of progesterone treatment (Bazer et al., 1979; Hansen et al., 1986; Moffatt et al., 1987b; Ing et al., 1989; Stephenson

and Hansen 1990; Leslie and Hansen 1991). Uterine fluid from pregnant (Stephenson et al., 1989) and progesterone treated ewes (Stephenson and Hansen, 1990; Hansen and Skopets, 1992) also contains activity inhibitory to lymphocyte proliferation and this activity has been ascribed to UTM-proteins (Skopets and Hansen, 1993). The following section is a review of biochemical properties of the UTM-proteins, hormonal regulation of their secretions and their lymphocyte regulatory properties.

Biochemical Properties

Uterine milk proteins are a pair of structurally related glycoproteins of $M_r$ = 55,000 and 57,000 with each having a pI of approximately 9.3 (Moffatt et al., 1987; Newton et al., 1989). A mixture of both UTM-proteins were estimated to have 5.6-5.7% N-linked carbohydrates (Hansen et al., 1987b). Analysis of the amino acid sequence indicates there are two sites for N-glycosylation (Ing and Roberts, 1989). Carbohydrate analysis indicates that CHO has been processed at least partially to complex-type forms (Hansen et al., 1987b). While differences between two forms of UTM-proteins could reflect differences in glycosylation, there is no evidence for differences in carbohydrate structure between the two forms of UTM-proteins (Hansen et al., 1987).

The two forms of UTM-proteins have a similar peptide structure as determined by peptide mapping analysis (Hansen et al., 1987a). The nucleotide sequence analysis indicates that the UTM-proteins contain two strong start signals (AUG) for translation at bases 1-3 and 16-18 (Ing and Roberts 1989). However, it is doubtful that the two

forms represent products of different start sites because N-terminal amino acid sequencing yielded only one amino acid at each position (Hansen et al., 1989; Ing and Roberts, 1989). The first 29 amino acids from the amino-terminus of mixed both UTM-proteins generally agrees with the inferred amino acid sequence from UTM-protein cDNA although two amino acids are not identical (Hansen et al., 1987a; Ing and Roberts 1989). In vitro, translation of RNA from pregnant endometrium resulted in two major products of $M_r$ = 47,000 and 55,000 which interacted with antibody to UTM-proteins (Ing and Roberts, 1989). However, pulse-chase labelling experiments indicated that the 55,000 and 57, 000 $M_r$ forms of UTM-proteins are preceded by a tissue form of $M_r$ 53,000 (Hansen et al., 1987a). Thus, it is unclear whether both forms of the proteins come from a common precursor or different mRNAs.

Uterine Milk Proteins Are Serpins

Based on the inferred animo acid sequences, UTM-proteins are members of the serpin superfamily of serine proteinase inhibitors (Ing and Roberts, 1989). UTM-proteins have 31% animo acid homology with baboon $\alpha_1$ antitrypsin and 27% homology with human $\alpha_1$ antichymotrypsin (Ing and Roberts, 1989). Also, the UTM-proteins have a putative reactive site characteristic of serpins that is at $Ala^{365}$ of C-terminal region. In addition, the predicted secondary structure of UTM-proteins is similar to the crystal structure of $\alpha_1$ antitrypsin (Ing and Roberts, 1989). As for another serpin, $\alpha_1$ AT, UTM-proteins can bind selectively to the Fc fragment of IgA (Hansen and Newton, 1988). The UTM-proteins do not inhibit any known serine

proteinase (Ing and Roberts, 1989) but are weakly inhibitory to aspartic proteinases such as pepsin (Mathialagan et al., 1995).

Uterine Milk Proteins are Pregnancy-Associated

UTM-proteins are the major pregnancy-associated proteins in uterine secretions. The messenger RNA for UTM-proteins can be detected in endometrium as early as day 14 of pregnancy and also at day 16 of the estrous cycle (Ing et al., 1989; Moffatt et al., 1987b). Furthermore, the proteins can be identified in uterine secretions from about day 16 of pregnancy but not in cycling animals. UTM-proteins become the major secretory proteins of the uterine endometrium after day 30 of pregnancy through to term (Moffatt et al., 1987b; Ing et al., 1989; Stephenson et al., 1989). UTM-proteins can be immunohistochemically localized to glandular and lumenal epithelium of the intercaruncular endometrium (Moffatt et al., 1987b; Stephenson et al., 1989a; Ing et al., 1989; Leslie and Hansen 1991).

The model of unilaterally-pregnant ewes was developed by Bazer et al. (1979) to increase the volume of uterine secretions for biochemical analysis. Large amounts of UTM-proteins can be obtained from the ligated uterine horn of unilaterally-pregnant ewes, especially during mid and late gestation. Approximately 200-1000 ml of fluid can be obtained from a single ewe and the UTM-proteins are present at concentrations of 5-10 mg/ml (Moffatt et al., 1987b). There is no difference in amount of protein synthesis or patterns of protein secretions from pregnant and nonpregnant uterine horns of unilaterally-pregnant ewes (Moffatt et al., 1987b; Stephenson et al., 1989a).

This result indicates synthesis and secretion of UTM-proteins are regulated by systemic effect of pregnancy and are not modified by a local effect of the conceptus.

Purification

As mentioned before, the UTM-proteins are the predominant proteins in uterine secretions of the unilaterally-pregnant ewe as determined by one or two-dimensional PAGE (Moffatt et al., 1987). The other major proteins in uterine secretions are basic polypeptides which react with monoclonal antibody to UTM-proteins (Leslie et al., 1990) and probably represent proteolytic breakdown products of UTM-proteins (Moffatt et al., 1987).

Isolation of UTM-proteins is typically achieved by ion-exchange chromatography using CM-cellulose and gel filtration (Moffatt et al., 1987). UTM-proteins purified by this procedure usually contain small amounts of low $M_r$ products of UTM-proteins. These increase upon storage of uterine fluid or purified preparation of UTM-proteins (Hansen and Liu, 1995). In addition, the UTM-protein are also prone to forming large molecular weight oligomers. Crude uterine fluid has a milky-white appearance and contains large amounts of colloidal or precipitated protein that represents primarily aggregates of UTM-proteins that can be dissolved by reducing agents (Moffatt et al., 1987).

Regulation by Progesterone

Progesterone is the major hormone regulating UTM-protein secretion. Progesterone treatment induces secretion of UTM-proteins and accumulation of its

mRNA in ovariectomized sheep (Hansen et al., 1986; Ing et al., 1989; Leslie and Hansen, 1991). Prolonged exposure to progesterone results in increased rates of secretion because of increasing number of cells producing UTM-proteins. UTM-proteins are first synthesized by epithelial cells in deeper areas of uterine glands and then the rest of glandular epithelium, and finally in the lumenal epithelium (Leslie and Hansen, 1991).

Immunoregulatory Properties

Several serpins regulate lymphocyte function. For example, human $\alpha_1$-AT and antithrombin III reduce lymphocyte responsiveness to PHA stimulation, inhibit the serine esterase-induced polyclonal activation and enhancement of B-cell function, and reduce natural killer cell activity (Hudig et al., 1981; Breit et al., 1985). Addition of $\alpha_1$-AT completely inhibits human lymphocyte surface proteolytic activity (Hudig et al., 1981). Furthermore, $\alpha_1$-AT can bind to lymphocytes and decreases [$^3$H]-thymidine incorporation into lymphocyte induced by B or T cell mitogens or by allogeneic cells (Bata and Revillard, 1981). In addition, a broad spectrum proteinase inhibitor $\alpha_2$ macroglobulin, inhibits NK cell and LAK cell functions, and blocks mitogen and alloantigen-induced T cell proliferation (Heumann and Vischer, 1988; Peterson et al., 1989).

UTM-proteins are also immunoregulators similar to $\alpha_1$ AT or other proteinase inhibitors. Uterine fluid of pregnant ewes inhibits mitogen and MLR-induced sheep PBL proliferation (Segerson et al., 1984; Zhang and Miller, 1989; Stephenson et al.,

1989) and also inhibits T cell antigens-induced mouse spleen cell proliferation (Stephenson et al., 1991). This lymphocyte-inhibitory activity in uterine fluid can be induced in ovariectomized ewes by progesterone (Hansen et al., 1986; Stephenson and Hansen, 1990; Hansen and Skopets, 1992). Furthermore, it has been found UTM-proteins from uterine fluid are responsible for most of the lymphocyte-inhibitory activity (Skopets and Hansen, 1993). Purified UTM-proteins inhibit PBL proliferation induced by PHA, Con A and MLR and *Candida albicans* antigen (Segerson et al., 1984; Zhang and Miller, 1989; Skopets and Hansen, 1993; Skopets et al., 1995). The concentration of UTM-proteins required to inhibit lymphocyte proliferation are high. Depending upon the assay and stimulants, 50-500 $\mu$g/ml of proteins are required for inhibition of lymphocyte proliferation (Skopets and Hansen, 1993). These ranges of concentrations are similar to concentrations of $\alpha_1$ -AT (Hudig et al., 1981; Breit et al., 1985).

The mechanism by which UTM-proteins inhibit lymphocyte activation is not clear. One possibility is that UTM-proteins may inhibit surface proteinase activity of lymphocytes like serpin $\alpha_1$-AT. For example, addition of 0.2 mg/ml $\alpha_1$-AT nearly completely suppressed the surface proteinase activity of human PBL (Bata and Revillard, 1981). The binding of $\alpha_1$-AT to lymphocytes may be the initial step in a series of regulatory events to inhibit lymphocyte function (Bata and Revillard 1981). As discussed later, the lymphocyte co-stimulatory molecule, CD26, which is serine dipeptidyle proteinase IV (DPPIV), may be a target molecule for inhibition. Another

possibility is that UTM-proteins bind non-specifically to lymphocytes to block interaction of lymphocyte receptor with their ligands. UTM-proteins can inhibit proliferation when addition of the proteins is delayed until 6 hours after addition of PHA (Skopets and Hansen, 1993), suggesting that the proteins act to inhibit PBL proliferation by binding of PHA to lymphocytes.

The subpopulations of lymphocytes inhibited by UTM-proteins are also not completely understood. UTM-proteins may have a minor effect on B cell activation because there is only limited inhibition of lymphocyte proliferation induced by T cell and B cell mitogen PWM (Skopets and Hansen, 1993). UTM-proteins have a strong inhibitory effect on T cell activation induced PHA and MLR (Skopets and Hansen, 1993; Skopets et al., 1995). However, UTM-proteins did not inhibit hypersensitivity reaction to PHA or *Mycobacterium tuberculosis* in sheep skin (Skopets et al., 1995). Therefore, it is important to understand how UTM-proteins influences uterine IEL subpopulations before the role of these proteins as immunosuppressive molecules that regulate maternal immune responses to the conceptus can be ascertained.

Are UTM-Proteins Binding Proteins ?

UTM-proteins may play a role in transporting targets from the uterus to the fetus. Several proteins have been identified in the pregnant uterus that function to transport nutrients to the fetus. For example, uteroferrin of the porcine uterus binds iron, causing the placenta to release its iron to the developing fetus (Roberts et al., 1986). Retinol binding protein is produced by the porcine and bovine uterus and is

involved in vitamin A transport to the fetus (Adams et al., 1981 and Thomas et al., 1992). Another example of a transport protein is $\alpha_2$ macroglobulin. This protein is found in the human and murine uterus during pregnancy (Gu et al., 1992; Bany and McRae, 1992; Rechberger and Woessner, 1994) and inhibits all four classes of proteinases (Williams et al., 1994). Also, $\alpha_2$ macroglobulin can bind various growth factors or cytokines, such as TGF-$\beta$, platelet-derived growth factor (PDGF), TNF, basic fibroblast factor (FGF), nerve growth factor (NGF), IL-1, IL-6 and insulin (Gettin and Crews, 1994). Some serpin proteins play a role in transporting steroid hormones or peptides by binding their targets (Schulze et al., 1994). For example, thyroxine binding globulin and cortisol binding globulin are transport proteins of the serpin family but do not have proteinase inhibitory activity (Pemberton et al., 1988).

It is not known whether UTM-proteins are transport proteins. These proteins do not bind calcium or $PGF_{2\alpha}$ (Moffatt et al., 1987). However, UTM-proteins cross the placenta and can be found in allantoic fluid (Moffatt et al., 1987; Zhang et al., 1989; Newton et al., 1989). Small amount of the proteins could be identified in chorioallantois using immunohistochemical staining (Moffatt et al., 1987a). UTM-proteins can bind to IgA (Hansen and Newton, 1988), this activity is not relevent to the placental transport because there is no movement of immunoglobulin across the ruminant placenta.

## Serpins and the Serine Proteinase CD26

### Serpin

The term serpin was coined by Carrel and Travis (1985) as an abbreviation (serine proteinase inhibitor) for members of a family of proteins which include $\alpha_1$-proteinase inhibitor ($\alpha_1$ PI) [also called $\alpha_1$-antitrypsin], $\alpha_1$-antichymotrypsin ($\alpha_1$ ACT), antithrombin III (AT III), heparin cofactor II (HC II), C1-inhibitor, protein C inhibitor and $\alpha_2$-antiplasmin ($\alpha_2$AP) (Huber and Carrell, 1989). These serpins share primary sequence homology and function as proteinase inhibitors or binding proteins (Andreasen et al., 1992).

Structure Serpins are single chain polypeptides containing a conserved domain of 370-390 residues. Within the structurally conserved domain, there is a region toward the C-terminal end termed the "reactive center region" or "reactive site loop (RSL)". Inhibitory serpins interact with their target proteinase at the RSL region. The RSL resides on the top of a protein scaffold and interacts with the underlying architecture to maintain a tightly constrained conformation (Enghild et al., 1994). The interaction between serpin and proteinase forms a stabilizing complex of enzyme-substrate. If stabilization does not occur rapidly enough, hydrolysis of the acyl intermediate occurs, leading to release of free proteinase and cleaved serpin (Gettins et al., 1993). This occurs for ovalbumin (Bruch et al., 1988). The cleaved serpin consist of three $\beta$-pleated sheets and several $\alpha$ helices.

Function The role of serpins in maintaining homeostasis is very important in many biological processes. Serpins are involved in the regulation of blood coagulation, fibrinolysis, inflammation, complement activation, immune responses and

antiproliferative or tumor-suppressing activity (Hudig et al., 1981; Carrell and Travis 1985; Masson and Tschopp 1988; Block 1990; Zou et al., 1994). Multiple regulatory functions of serpins are summarized in the Table 2-1.

Table 2-1 Summary of some serpins and their functions

| Serpins | Target enzyme | Function | Ref. |
|---|---|---|---|
| $\alpha_1$-PI; $\alpha_1$ AT | Neutrophil elastase | Extracellular matrix remodeling; lymphocyte inhibitory | Potempa et al., 1994; Breit et al., 1985 |
| Maspin | ? | Tumor suppression | Zou et al., 1994 |
| UTM-proteins | ? | Lymphocyte inhibitory | Hansen and Liu, 1994 |
| Leukocyte inhibitor | neutrophil elastase | Intracellular proteolysis | Dubin et al., 1992 |
| CrmA | ICE | Modulation of inflammatory response | Komiyama et al., 1994 |
| PAI-2 | u-PA, t-PA | Cell migration; Fibrinolysis | Andreasen et al., 1990 |
| HSP47 | ? | Protein folding | Wang, 1992 |
| TBG | ? | Hormone transport | Pemberton et al., 1988 |
| AT III | Thrombin | Blood coagulation | Pratt et al., 1993 |
| C1-inh | C1 esterase | Complement cascade | Travis and Salvesen ., 1983 |
| Angiotensinogen | ? | Blood pressure | Doolittle, 1983 |
| PN-1 | Thrombin | Cell differentiation | Monard, 1989 |

Abbreviations used are in this table. AT III, Antithrombin III; TBG, thyroxine binding globulin; PN-1, proteinase nexin-1; C1-inh, C1-inhibitor; PAI-2, plasminogen activator inhibitor-2; PI, proteinase inhibitor; AT, antitrypsin. ICE, interleukin-1$\beta$ converting enzyme; t-PA, tissue-type plasminogen activator; u-PA, urine plasminogen activator; Crm A, cytokine response modifier A.

CD26

CD26 is a highly glycosylated type II membrane sialoglycoprotein that has been found in many organs and cell types (Fleischer, 1994) including human placenta (Puschel et al., 1982). Recently, CD26 has been cloned from several species

(Marguet et al., 1992; Ogata et al., 1989; Darmoul et al., 1992; Tanaka et al., 1992). The sequences show a surprising conservation between different species with up to 98% identity between mammalian CD26 cDNAs and 36% between mammalian and yeast CD26 (Fleischer, 1994).

Structure The protein is anchored to the plasma membrane through hydrophobic amino acids in the amino-terminal region between residues 7 and 26. Most of the protein is located extracellularly, with a cytoplasmic tail of only six amino acids in length (Tanaka et al., 1992). The extracellular domain of human CD26 consists of 738 carboxyl-terminal amino acids and is divided into three regions; an amino-terminal glycosylated region (residues 29 to 323), a cysteine-rich middle section (residues 324-551), and a carboxyl-terminal region (residues 552-766). The amino-terminal region contains eight of the 10 potential attachment sites for N-linked glycans and one of the 12 cysteine residues. The carboxyl-terminal region contains G-W-S-Y-G, a putative catalytic center. Digestion of hepatocytes with papain leads to cleavage of CD26 near the cell surface and release of a soluble form of CD26 with the new N-terminal at amino acid 35 (Fleischer, 1994).

Function CD26 is also called DPPIV, because it is a serine proteinase that possesses proteolytic activity against polypeptides in which proline is at the penultimate position (Walter et al., 1980). As a result, CD26 removes dipeptides from the N-terminus. Many biologically active polypeptides have the sequence X-Pro at the N-terminus and can be potential substrates for CD26. These include substance P, chorionic

gonadotropin, IL-1$\beta$, IL-2, TNF-$\beta$ and G-CSF (Fleischer, 1994).

CD26 is a costimulory protein in the process of T cell activation. The reaction of specific peptide antigen or antibodies with TCR complex is generally unable to induce T cell proliferation and lymphokine production. T cells require coreceptors or accessory molecules expressed on the T cell surface to allow activation (Geppert et al., 1990). CD26 exhibits costimulatory activity (Dang et al., 1990) and its expression on lymphocytes is enhanced following T-cell activation (Fox et al., 1984). Moreover, costimulation of CD26 leads to the activation of all functional programs of the T cells including proliferation, IL-2 production, cytotoxicity and granule exocytosis (Fleischer et al., 1988; Dang et al., 1990; Mattern et al., 1993; Bristol et al., 1992). The molecular mechanisms by which CD26 regulates T cell activation are still not clear. The CD26 pathway probably requires the components of the TCR/CD3 complex, which mediates signal transduction of T cell activation and leads to the same pattern of tyrosine phosphorylation as triggering through the TCR (Fleischer et al., 1988; Torimoto et al., 1991). CD26 is also associated with another molecule that is important for T cell biology, the 41 kDa adenosine deaminase (ADA) protein, the deficiency of which results in SCID in humans. The extracellular domain of CD26 binds directly to ADA and thereby transports it to the T cell surface (Kameoka et al., 1993). Also, CD26 can bind to components of the extracellular matrix, such as fibronectin and collagen (Hanski et al., 1985) and may be involved in the interaction of cells and the extracellular matrix.

In addition, enzymatic activity plays a role in T cell proliferation because inhibition of CD26 enzymatic activity blocks the costimulatory activity of CD26 (Tanaka et al., 1993). Indeed, specific inhibitors of DPPIV have been shown to block T-cell activation by antigens (Flentke et al., 1991) and by lectins (Schon et al., 1987) and suppress an immune responses *in vivo* (Kubota et al., 1992). Mutant CD26 ($DPPIV^-$)-transfected Jurkat cells produce IL-2 much less than do wild-type CD26 ($DPPIV^+$) (Tanaka et al., 1993). Thus, DPPIV activity of CD26 functions to augment the cellular responses of T cells to external stimuli through enhancing IL-2 production.

## Summary

Interactions between the maternal system and the conceptus take place during pregnancy in many species. Maternal immune responses to the alloantigeneic conceptus may contribute to pregnancy failure or could promote placental function through cytokine secretion from activated T or NK cells. Several mechanisms are involved in regulating the interactions between the maternal immune system and the conceptus. First, the feto-maternal unit or progesterone can direct T helper cells to produce Th2-type cytokine production and enhancement of embryonic growth. Second, there are several types of immunosuppressive regulators at the feto-maternal interface that regulate activation of uterine lymphocytes. Third, the expression of MHC class I molecules on trophoblast may provide maternal NK and T cells with inhibitory signals that protect the conceptus from attack by the maternal immune system in mice and other species in which trophoblast expresses MHC class I

antigens. There were three major objectives of the experiments in this dissertation. The first objective was to test the mechanisms by which UTM-proteins regulate lymphocyte function in order to understand how UTM-proteins block lymphocyte activation. Specific goals were to evaluate whether UTM-proteins specifically bind to lymphocytes and whether the binding is mediated by receptor-ligand interaction; to test whether this interaction generates an inhibitory pathway that blocks lymphocyte activation; and to understand the possible interaction between UTM-proteins and CD26; The second major objective was to study whether endometrial serpin-like proteins regulate activation of NK cells. Specific goals were to evaluate effects of UTM- proteins on NK cell activity in sheep and mice in vitro and in vivo and to examine effects of UTM-proteins on poly(I).poly(C)-induced embryonic degeneration in mice. The third major objective was to characterize changes in activation markers on uterine $\gamma\delta$ $TCR^+$ IEL during pregnancy and evaluate whether pregnancy-associated changes in uterine $\gamma\delta$ $TCR^+$ IEL are due to systemic effects of pregnancy (including effects of UTM-proteins) or to the local effect of the conceptus.

# CHAPTER III
# MECHANISMS FOR INHIBITION OF LYMPHOCYTE ACTIVATION BY UTM-PROTEINS

## Introduction

During pregnancy or following progesterone treatment, the endometrium of the sheep synthesizes a pair of related glycoproteins of 55 kDa and 57 kDa called the UTM-proteins (Moffatt et al., 1987a; Moffatt et al., 1987b; Leslie and Hansen, 1991). The amino acid sequence of UTM-proteins indicates homology with several members of the serpin superfamily (Ing and Roberts, 1989); The greatest homology is with baboon $\alpha_1$-antitrypsin (31%) and human $\alpha_1$- antichymotrysin (27%). Like other serpins, $\alpha_1$-antitrypsin (Hudig et al., 1981; Breit et al., 1985) and antithrombin (Masson et al., 1988), and the broad spectrum proteinase inhibitor, $\alpha_2$-macroglobulin (Heumann et al., 1988; Petersen et al., 1989; Borth et al., 1994), the endometrial serpin like-proteins possess immunosuppressive activity. In particular, UTM-proteins inhibit mitogen and antigen-induced activation of sheep and mouse lymphocytes (Segerson et al., 1984; Stephenson et al., 1989a; Zhang and Miller, 1989; Skopets and Hansen, 1993), reduce sheep and mouse natural killer cell activity in vitro and in vivo (Chapter IV), and reduce antibody production in sheep immunized with ovalbumin (Skopets et al., 1995). It has been suggested that the major role of UTM-proteins during pregnancy is to regulate maternal immune responses in the uterus to

allow survival of the alloantigeneic conceptus (Hansen and Liu, 1994). Indeed, progesterone can prolong skin graft survival in the uterus (Hansen et al., 1986) and reduce numbers of endometrial lymphocytes (Gottshall and Hansen, 1993), suggesting progesterone affects lymphocyte traffic in the uterus. UTM-proteins could alter expression of adhesion molecules on the lymphocyte surface to mediate this effect.

The mechanisms by which UTM-proteins regulate lymphocyte function are not clear. It is likely that UTM-proteins modulate lymphocyte function by binding to a cell surface receptor. Such a receptor could be distinct for UTM-proteins or be analagous to other receptors, such as the serpin-enzyme complex receptor (SECR) (Permutter et al., 1990; Joslin et al., 1991). There are several steps at which UTM-proteins could block lymphocyte proliferation. Generally, lectins or antigens stimulate lymphocytes through tyrosine kinase dependent activation of phospholipase C followed by protein kinase C activation and Ca++ mobilization (Weiss and Littman, 1994). UTM-proteins could interfere with this cascade. Alternatively, UTM-proteins could affect IL-2 regulated proliferation by affecting IL-2 receptor expression in response to stimulation or by blocking IL-2-dependent stimulation. The later effect is exerted by $\alpha_2$-macroglobulin (Heumann and Vischer, 1988). The purpose of the present study was to determine how UTM-proteins inhibit lymphocyte activation by determining effects of UTM-proteins on signal transduction pathways, lectin binding to lymphocytes and expression of several cell surface markers on $\gamma\delta$ $TCR^+$ and $\gamma\delta$ $TCR^-$ cells. Additional goals were to determine whether binding of UTM-proteins to

lymphocytes is required for its function and to further characterize whether such binding was via SECR or other binding sites.

## Materials and Methods

### Reagents

Biotin hydrazide was from Pierce (Rockford, IL), sodium meta-periodate and $\beta$-mercaptoethanol were from Fisher (Pittsburggh, PA), 4-(2-aminoethy)-benzenesulfonyl floride hydrochloride (AEBSF) was purchased from Boehringer Mannheim (Indianapolis, IN). Allergenic extract from *Candida albicans* was from Greer Laboratories (Lenoir, NC). [$^3$H-methyl] thymidine (specific activity 5 Ci/mmol) was from Amersham (Costa Mesa, CA). Defined horse serum was obtained from Hyclone (Logan, UT) and recombinant human IL-2 was purchased from Genzyme (Boston, MA). Molecular standards were purchased from Bio-Rad (Hercules, CA). Immobilon-P membrane was from Millipore (Bedford, MA). Other materials were from Sigma (St Louise, MO).

### Antibodies

Hybridomas producing antibodies to $\gamma/\delta$ TCR (86D), CD44 (25-32), CD29 (47), Mel-14 (Du129), CD8 (7C2) and CD4 (17D) were purchased from the European Collection of Animal Cell Culture, Salisbury, UK. Anti-CD25 monoclonal antibody (9-14) was kindly provided by Dr. Andrew D. Nash, Centre for Animal Biotechnology, School of Veterinary Science, The University of Melbourne, Parkville, Australia. Antibody (HL 708) to UTM-proteins was described previously (Leslie and

Hansen, 1991). For each antibody, ascites were prepared from Balb/c mice. FITC-conjugated sheep antibody to mouse whole Ig was from Sigma. Except for anti-CD25 and anti-UTM-proteins, which were used without purification, antibodies were purified using Protein A-agarose affinity chromatography.

Purification of Uterine Milk Proteins

Uterine fluid obtained from the ligated uterine horn of unilaterally pregnant ewes (Bazer et al., 1979) was dialyzed against 10 mM Tris-HCl, pH 8.2. After dialysis, the fluid was loaded onto a CM-Sepharose column (15 x 2.5 cm) pre-equilibrated in the same buffer, and the basic proteins containing UTM-proteins were eluted with 10 mM Tris-HCl, pH 8.2/0.3 M NaCl. Proteins were further purified by FPLC using a Mono S 5/5 column with 50 mM Bicine buffer (pH 8.7) as eluted with a linear gradient of 0-1.0 M NaCl and at a flow rate of 1 ml/min. Samples were then dialyzed against 10 mM Tris-HCl (pH 8.2/0.33 M NaCl) and further purified using a Superdex 200 column (16 mm x 62 cm) with 10 mM Tris-HCl (pH 8.7/0.33M NaCl) and 0.5 ml/min flow rate. Purity was determined by SDS-PAGE using 12.5% polyacrylamide gels and the method of Laemmli (1970). Additionally, the final preparation was analyzed by immunobloting using a monoclonal antibody against UTM-proteins. Proteins were electroeluted onto an Immobilon-P membrane in 20 mM Tris-glycine buffer at 200 mA overnight at 4 C. UTM-proteins were localized on the immunoblots by incubation with HL708 ascites (1:1500 dilution) or control ascites (Sigma) for 1 h and stained with goat anti-mouse Ig (whole molecule) alkaline

phosphatase conjugate (Sigma) for 1 h. Alkaline phosphatase was diluted by fast red/napthol phosphate.

Biotinylation

Protein A agarose-purified monoclonal antibody (2 mg/ml) was reacted with 20 mM sodium meta-periodate in 0.1 M sodium acetate buffer (pH 5.5). After oxidation for 20 min on ice, the IgG was dialyzed against 0.1 M sodium acetate buffer (pH 5.5) overnight. Biotin hydrazide was added to the IgG solution at a final concentration of 1.2 mg/ml. After 2 h at room temperature, biotinylated IgG was dialyzed twice against 0.1 M sodium acetate (pH 5.5) and once against DPBS. Samples were filtered through 0.2 $\mu$m filters and aliquots stored at -20 C. UTM-proteins were biotinylated similarly except that oxidation was with 30 mM sodium-meta-periodate and biotin hydrazide was reacted for 1 h at room temperature.

Determination of Protein Concentration

Protein concentration was determined using the BCA assay with BSA as a standard (Smith et al., 1985).

Preparation of Peripheral Blood Lymphocytes

Peripheral blood lymphocytes (PBL) were purified from jugular venous blood of Rambouillet ewes as described previously (Skopets et al., 1993; Chapter IV). For flow cytometry, purified cells were resuspended to a concentration of 1 x $10^7$/ ml in staining buffer [1% (w/v) BSA and 0.1 %(w/v) EDTA in DPBS]. For proliferation studies, purified cells were resuspended to 1 x $10^6$/ml in culture medium.

Inhibition of PHA, PMA, IL-2, and Antigen-Induced Lymphocyte Proliferation

Peripheral blood lymphocytes were resuspended (1 x $10^6$/ml) in a medium consisting of TCM-199 containing 5% (v/v) horse serum, 5 $\mu$M $\beta$-mercaptoethanol, 0.2 mM pyruvate, 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin and 2 mM extra L-glutamine. Lymphocytes (1 x $10^5$) were incubated with 4 nM PMA, 4 U IL-2, or 2 $\mu$g PHA and with UTM-proteins (5 to 30 $\mu$g), ovalbumin (30 $\mu$g) or ovine serum albumin (30 $\mu$g) in a total volume of 110 $\mu$l (100 $\mu$l cell medium + 10 $\mu$l DPBS). After 12 or 60 h of culture at 37 C and 5% $CO_2$, 0.4 $\mu$Ci [$^3$H]thymidine was added in a volume of 50 $\mu$l culture medium. For antigen-induced lymphocyte proliferation, cells (1 x $10^5$ /well) were incubated with 1:3 dilution of *C. albicans* extract and various amounts of either UTM-proteins or lysozyme in a total volume of 120 $\mu$l (100 $\mu$l cell medium + 20 $\mu$l DPBS). After 5 days culture at 37C and 5% $CO_2$, 0.4 $\mu$Ci [$^3$H]thymidine were added in a volume of 50 $\mu$l culture medium. For both experiments, cells were harvested 12 h later using a Brandel cell harvest (Gaithersburg, MD) and radioactivity incorporated into DNA determined by scintillation spectrometry.

In both experiments, treatments were prepared in triplicate for each assay and replicated using PBL from four separate ewes.

Expression of Cell Surface Receptors

Peripheral blood lymphocytes (5 x $10^6$ in 5 ml) were cultured in RPMI-1640 containing 10% fetal calf serum, 1% (w/v) extra L-glutamine, 100 IU/ml penicillin

and 100 $\mu$g/ml streptomycin at 37 C° in 5% $CO_2$. Cells received treatments of PHA ($\pm$ 10 $\mu$g/ml) and UTM-proteins ($\pm$ 200 $\mu$g/ml). After 20 h incubation, cells were washed with staining buffer and used for two-color immunofluorescence analysis using antibody to $\gamma/\delta$ TCR and either CD25, CD44, CD29 and Mel 14. Cells (1 x $10^6$) in 100 $\mu$l same staining buffer were incubated with the primary antibody for 30 min on ice, then washed in staining buffer and incubated with 10 $\mu$l goat anti-mouse Ig G (whole molecule) coupled to FITC for 30 min on ice. Cells were washed, incubated with 2 ng biotinylated antibody against $\gamma/\delta$ TCR in 100 $\mu$l staining buffer for 30 min on ice and then incubated with 5 $\mu$g streptavidin-PE in 100 $\mu$l staining buffer for 10 min on ice. Immunofluorescent analysis was performed using a FACScan flow cytometer. The experiment was replicated using cells from four separate ewes.

Effect of UTM-proteins on Binding of PHA and Con A to Lymphocytes

Peripheral blood lymphocytes (1 x $10^6$) were incubated on ice in 100 $\mu$l staining buffer with 5 $\mu$g PHA-FITC or Con A-FITC and either 0, 5 or 20 $\mu$g UTM-proteins or 20 $\mu$g OSA. After 1 h, cells were washed twice and binding of FITC to lymphocytes was determined by FACScan. The experiment was replicated using cells from three separate ewes.

Preparation of Serpin-Enzyme Complex

To prepare trypsin-antitrypsin, bovine trypsin (1 mg) and 4 mg bovine $\alpha_1$-antitrypsin were incubated in 2 ml of DPBS containing 2% bovine serum albumin, 3 mM $CaCl_2$ and 3 mM $MgCl_2$ for 30 min at 37 C. Finally, AEBSF was added to a

5 mM concentration. Thrombin-antithrombin III complex was prepared using a modified procedure of Takeya et al. (1994). Three units of human antithrombin III and 3 units of human thrombin were incubated in 10 mM Tris-HCl (pH 8.2) containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$ at room temperature for 30 min.

Analysis of Binding of UTM-Proteins to Lymphocytes

Peripheral blood lymphocytes (1 x $10^6$) were incubated with different concentrations of biotinylated UTM-proteins (Bt-UTMP) (3-50 $\mu$g per tube) for 1 h on ice. Binding was performed in the presence or absence of various inhibitors (for example, unlabeled UTM-proteins or trypsin-antitrypsin) or control proteins. Cells were incubated for 1 h on ice. After incubation, cells were washed twice in staining buffer, incubated with 5 $\mu$g streptavidin-phycoerythrin (PE) in 100 $\mu$l of staining buffer for 30 min on ice, washed with staining buffer, and then analyzed for binding by a FACScan flow cytometer.

Inhibition of Binding to Lymphocytes by Heparin

An experiment was done using PBL (1 x $10^6$ cells in 100 $\mu$l staining buffer) from three ewes in which heparin and Bt-UTMP were added simultaneously for 60 min on ice. In addition, peripheral blood lymphocytes from six separate ewes were preincubated with different amounts of heparin (3, 6, 9, 12 units) for 30 min on ice. After washing twice in staining buffer, cells were incubated with 2 $\mu$g Bt-UTMP in 100 $\mu$l staining buffer for 60 min on ice. Following this step, cells were washed and 5 $\mu$g of streptavidin-PE was added. After 30 min, cells were washed, resuspended

in 1 ml staining buffer and used for the binding analysis by a FACScan.

Effect of Heparin on Inhibition of PHA-Induced Lymphocyte Proliferation by UTM-Proteins

Peripheral blood lymphocytes were resuspended at 1 x $10^6$/ml in TCM 199 containing 5% horse serum, 100 $\mu$g/ml streptomycin, 100 IU/ml penicillin, and 2 mM extra L-glutamine. Cells (1 x $10^5$) were placed in wells of 96-well plates in triplicate and incubated in the presence of 2 $\mu$g PHA (+, -), 50 $\mu$g UTM-proteins (+, -) and different amounts of heparin (0, 3, 6, 9, 12 units) in a total volume of 120 $\mu$l for 72 h at 37 C and 5% $CO_2$. After 60 h of incubation, 0.4 $\mu$Ci [$^3$H]thymidine were added in a volume of 50 $\mu$l culture medium. Cells were harvested 12 h later and the radioactivity incorporated in DNA determined by scintillation spectrometry. The experiment was replicated using cells from six separate ewes.

Statistical Analysis

All data were analyzed by least squares analysis of variance according to the General Models Program of the Statistical Analysis System (SAS, 1989). Ewe (i.e., lymphocyte donor) was considered as a random effect and other treatments were considered fixed effects. Duncan's test and orthogonal contrasts were used to determine differences between levels when there were more than 2 levels of a main effect. Dose-response relationships were determined using orthogonal polymonomial procedures. All data are presented as least-squares means $\pm$ SEM.

## Results

### Purification of UTM-Proteins

The purification of UTM-proteins from uterine secretions was performed using conventional ion-exchange, FPLC ion-exchange and gel filtration chromatography. A representative purification result is shown in Figure 3-1. Basic proteins that bound to CM-Sephrose at pH 8.2 were batch-eluted from the column by 0.33 M NaCl. Subsequently, further resolution of this material by FPLC using Mono-S revealed one major peak of basic proteins after linear gradient salt elution. These materials were pooled and a single major peak was resolved when using a Superdex 200 column. Analysis of protein patterns from samples collected

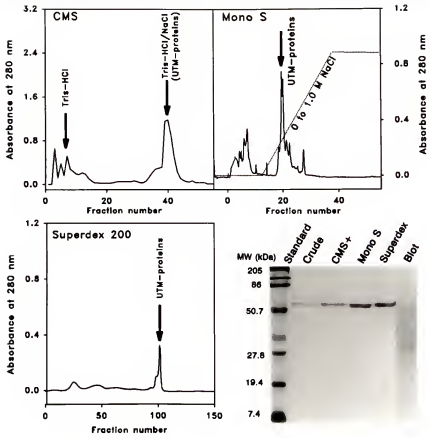


Figure 3-1. Purification of UTM-proteins from uterine fluid of unilaterally pregnant ewes. Uterine fluid was dialyzed against 10 mM Tris-HCl (pH 8.2) and basic proteins binding to CM-Sepharose were eluted with 0.33 M NaCl (left upper panel). Subsequently, the basic protein fraction was loaded on a Mono S column and basic proteins were eluted with a linear NaCl concentration gradient in 50 mM Bicine buffer, pH 8.7, (upper right panel). The UTM-protein containing fractions were further purified using Superdex 200 (lower left panel). Purification was determined by SDS-PAGE (lower right panel). Lanes 1-5 were stained with Coommassie Blue staining. Note, UTM-proteins exist primarily a pair of proteins of $M_r$ = 55,000 and 57,000. UTM-proteins are major proteins throughout purification. The last lane represents an immunoblot in which proteins from the Superdex 200 fraction were reacted with a monoclonal antibody to UTM-proteins.

oughout purification by SDS-PAGE and Coommassie blue staining revealed that final preparation of UTM-proteins appeared as purified mixture of 55 and 57 kDa forms of the proteins. Nevertheless, several low molecular weight forms of UTM-

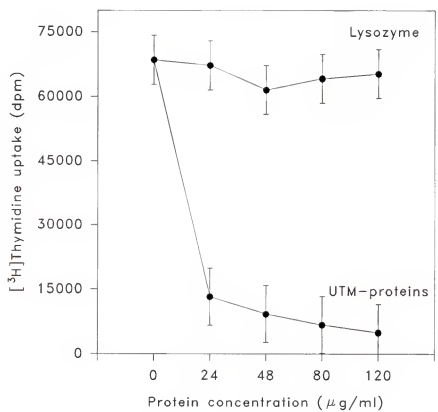


Figure 3-2. Inhibition of antigen-induced proliferation of PBL by UTM-proteins. Peripheral blood lymphocytes were cultured with *C. albicans* extract in the presence of various amounts of UTM-proteins. On day 5 of culture, [$^3$H]thymidine was added and radioactivity incorporated into DNA was determined after 12 h. Results are least-squares means $\pm$ SEM of results from four ewes. Antigen-induced proliferation was affected by dose of UTM-proteins ($p < 0.001$).

proteins remain present in the final preparation. While not seen by Coommassie Blue staining, these can be identified by immunobloting using a monoclonal antibody that recognizes the low molecular forms of UTM-proteins.

Inhibition of Antigen-Induced Proliferation by UTM-proteins

Culture of lymphocytes with allergenic extract of *C. albicans* caused an increase in incorporation of [$^3$H]thymidine from an average of 3375 dpm to 68528

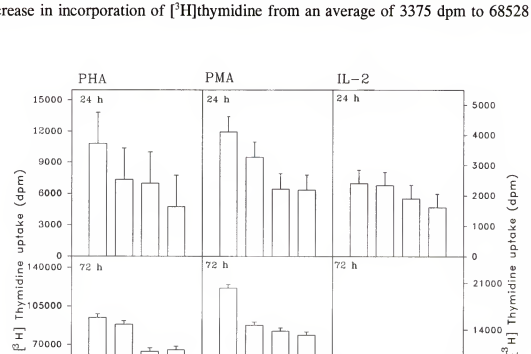


Figure 3-3. Effect of UTM-proteins on proliferation of lymphocytes induced by PHA, PMA and IL-2. For PHA-induced lymphocyte proliferation, [$^3$H]thymidine uptake was affected by time ($p < 0.05$), time x concentration ($p < 0.05$) and concentration of UTM-proteins ($p < 0.05$). Effects for PMA-treated cells were by concentration ($p < 0.05$) and time x concentration ($p < 0.001$). Interleukin-2-induced lymphocyte proliferation was affected by time ($p < 0.05$) only. Results are least-squares means $\pm$ SEM from four separate ewes.

dpm. As shown in Figure 3-2, incubation of lymphocytes with UTM-proteins

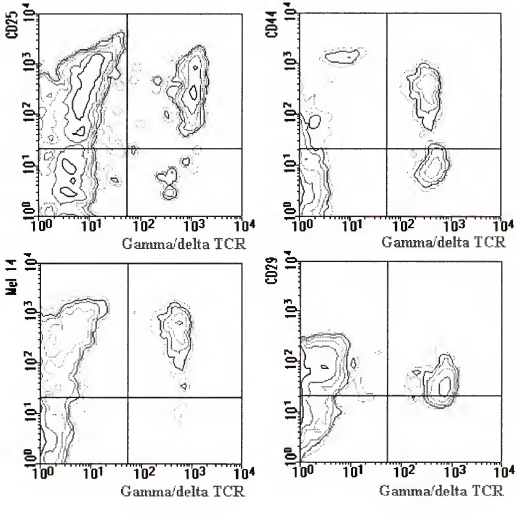


Figure 3-4. Representative two-color flow cytometry patterns for expression of CD25, CD44, CD29 and Mel-14 on $\gamma/\delta$ $TCR^+$ cells and $\gamma/\delta$ $TCR^-$ cells.

caused a large reduction in [$^3$H]thymidine incorporation ($p < 0.001$). At the lowest concentration tested, 24 $\mu$g/ml, incorporation of [$^3$H]thymidine was inhibited by 95%. In contrast, lysozyme, which was used as a control protein, had no effect on [$^3$H]thymidine incorporation of antigen-stimulated lymphocytes.

Effects of UTM-Proteins on PHA, IL-2 and PMA-Induced Lymphocyte Proliferation

UTM-proteins reduced [$^3$H]thymidine incorporation of lymphocytes stimulated by PHA ($p < 0.01$) and PMA ($p < 0.01$), but had no significant effect on the [$^3$H]thymidine incorporation of IL-2-treated PBL (Figure 3-3). Neither 300 $\mu$g/ml

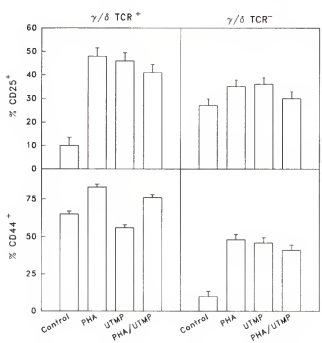


Figure 3-5 Effect of UTM-proteins on expression of CD25, CD44 on $\gamma/\delta$ TCR$^+$ and $\gamma/\delta$ TCR$^-$ lymphocytes. CD25 was affected by PHA ($p = 0.08$ for $\gamma/\delta$ TCR$^+$ cells, no significant for $\gamma/\delta$ TCR$^-$) and UTM-proteins ($p = 0.02$ for $\gamma/\delta$ TCR$^+$ cells, no significant for $\gamma/\delta$ TCR$^-$). CD44 expression on $\gamma/\delta$ TCR$^+$ cells was affected by PHA ($p = 0.08$ ), UTM-proteins ($p=0.05$) and PHA x UTM-proteins ($p = 0.07$). Results are least-squares means $\pm$ SEM from four separate ewes.

OVA or OSA blocked PHA or PMA-induced proliferation (results not shown).

Effect of UTM-Proteins on Expression of CD25, CD44, CD29 and Mel-14

Representative flow cytometry patterns are shown in Figure 3-4. Using two-color cytometry with antibodies to CD25, CD44, CD29 and Mel-14 as the first color and antibody to $\gamma/\delta$ $TCR^+$ cells as second color, PBL could be divided into four population on basis of antibody reactivity towards $\gamma/\delta$ TCR and CD25, CD44, CD29 and Mel-14 staining. Effects of UTM-proteins on PHA-induced changes in CD25, CD44, CD29 and Mel-14 expression on $\gamma/\delta$ $TCR^+$ and $\gamma/\delta$ $TCR^-$ cells were determined. Neither PHA nor UTM-proteins affected the percentage of cells staining for Mel-14 (58% positive for $\gamma/\delta$ $TCR^+$ and 56% positive for $\gamma/\delta$ $TCR^-$

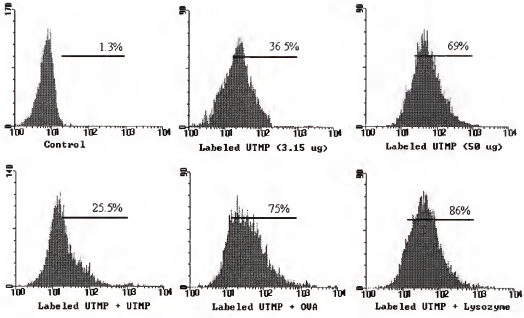


Figure 3-7 Representative patterns of binding of biotinylated UTM-proteins to PBL as resolved by one color flow cytometry. Figures demonstrate UTM-proteins binding to lymphocytes in the absence or presence of unlabeled UTM-proteins or control proteins. Peripheral blood lymphocytes (1 x $10^6$ in 200 $\mu$l) were incubated with labeled UTM-proteins (3.15 or 50 $\mu$g) or 50 $\mu$g labeled UTM-proteins plus 50 $\mu$g unlabeled UTM-proteins or lysozyme. Y axis shows numbers of lymphocytes bound to UTM-proteins; X axis shows intensity of fluorescence. The percentages in each figures represent the proportion of cells that stained positive (i.e., intensity was greater than the threshold intensity determined for PBL without Bt-UTMP.

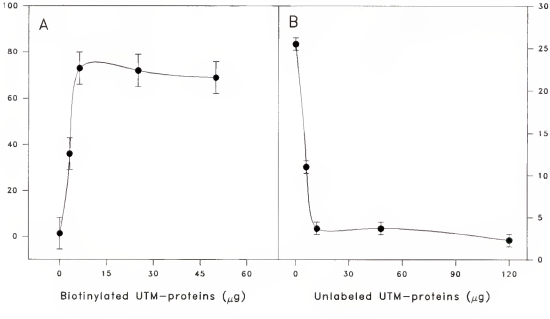


Figure 3-8 Binding of Bt-UTMP to PBL. Panel A. Peripheral blood lymphocytes (1 x $10^6$) were incubated with different concentrations of Bt-UTMP in 200 $\mu$l staining buffer for 1 h on ice. Binding was concentration-dependent ($p < 0.001$). Results are the least-squares means $\pm$ SEM from four ewes. Panel B. Competition for binding of Bt-UTMP to lymphocytes by unlabeled UTM-proteins. Lymphocytes (1 x $10^6$) were incubated with 3.15 $\mu$g Bt-UTMP for 1 h on ice with various amounts of unlabeled UTM-proteins in a total volume of 250 $\mu$l. The effects of concentrations were significant ($p < 0.001$). Results are the least-squares means $\pm$ SEM from three ewes.

cells, respectively). PHA decreased expression of CD29 for $\gamma/\delta$ $TCR^+$ (29% vs 46%,

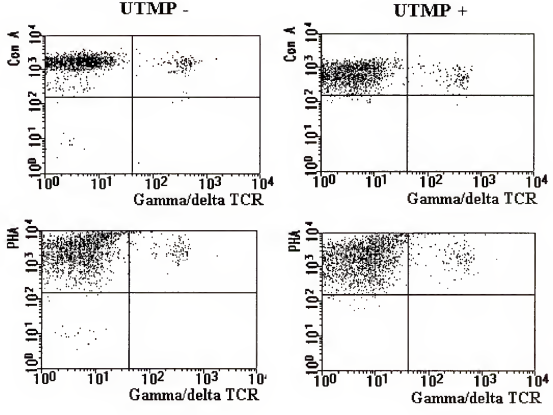


Figure 3-6. Representative results of flow cytometric analysis testing effects of UTM-proteins on binding of PHA or Con A to lymphocytes. Purified PBL were incubated with PHA-conjugated FITC or Con A-conjugated FITC with or without UTM-proteins. After washing, cells were stained by biotinylated antibody to $\gamma/\delta$ TCR and streptoavidin-PE. Immunofluorescence analysis was carried out by using FACScan. UTM-proteins did not reduce the percentage of $\gamma/\delta$ $TCR^-$ or $\gamma/\delta$ $TCR^+$ lymphocytes staining positive for PHA or Con A.

p = 0.07) and $\gamma\delta$ TCR$^-$ (22% vs 40%, p = 0.02), but there was no effect of UTM-proteins on CD29. PHA increased expression of CD25 and CD44 on both $\gamma/\delta$TCR$^+$ and $\gamma/\delta$ TCR$^-$ cells (Figure 3-5). Surprisingly, UTM-proteins increased expression of CD25 in the absence of PHA for both $\gamma/\delta$ TCR$^+$ and $\gamma/\delta$ TCR$^-$ cells.

Effect of UTM-proteins on Binding of PHA and Con A to PBL

The effects of UTM-proteins on PHA and Con A binding to the $\gamma/\delta$TCR$^+$ and $\gamma/\delta$ TCR$^-$ cells were determined to evaluate whether UTM-proteins could affect cells staining positive for PHA and Con A. Over 99% of $\gamma/\delta$TCR$^+$ cells and 96% of $\gamma/\delta$ TCR$^-$ cells were bound by PHA or Con A in the presence of 50 or 200 $\mu$g/ml UTM-proteins (Figure 3-6).

Binding of UTM-Proteins to Lymphocytes

The binding of Bt-UTMP to lymphocytes was determined by flow cytometry. A representative result is shown in Figure 3-7. The threshold for intensity of positive staining cells was determined by evaluating fluorescent intensity of cells without Bt-UTMP. As shown for a representative experiment (Figure 3-7) or for average results from replicated experiments (Figure 3-8), binding of Bt-UTMP to PBL was dose-dependent (p= 0.001) and saturable (Figure 3-8A). Also, unlabeled UTM-proteins caused competitive inhibition of binding of Bt-UTMP to lymphocytes (Figure 3-8B).

Specificity of Binding of UTM-Proteins to PBL

Data in Figure 3-8 represent results of an experiment to test specificity of

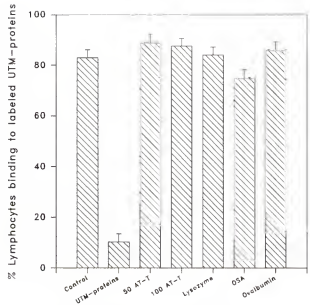


Figure 3-9 Specificity of UTM-proteins to lymphocytes. Lymphocytes (1 x $10^6$) were incubated with 50 $\mu$g of Bt-UTMP for 1 h on ice in 250 $\mu$l staining buffer in the absence of other proteins (control), presence of 300 ng trypsin or 50 $\mu$g unlabeled UTM-proteins (UTM-proteins), ovine serum albumin (OSA), ovalbumin or lysozyme or with 50 or 100 $\mu$g $\alpha_1$ AT-trypsin complex (AT-T). Control differs from trypsin ($p < 0.001$) and UTM-proteins ($p < 0.001$). Results are least-squares means $\pm$ SEM of results from two animals.

UTM-protein binding to lymphocytes. While binding of labeled UTM-proteins was inhibited by unlabeled UTM-proteins, there was no inhibition caused by other proteins including lysozyme, OSA and ovalbumin. The effects of a complex of trypsin -$\alpha_1$AT on binding was evaluated because this complex can inhibit serpins that bind cells through a SECR (Permutter et al., 1990). However, neither antitrypsin-trypsin complex (Figure 3-9) or thrombin-antithrombin (results not shown) had an effect on binding of UTM-proteins. Thus, UTM-proteins binding is not mediated through the SECR.

Effect of Heparin on Bt-UTMP Binding and Anti-proliferative Activity

Effects of heparin on binding of Bt-UTMP and lymphocyte-inhibitory activity are shown in Figure 3-10. The binding of Bt-UTMP to PBL was reduced in a dose-dependent manner by the presence of heparin (Figure 3-10A). The inhibitory effect of heparin on binding was effective when cells were preincubated with heparin and then washed before addition of Bt-UTMP (Figure 3-10B). Furthermore, heparin partly reduced the inhibitory effect of UTM-proteins on PHA-induced lymphocyte proliferation (Figure 3-10C). For example, 500 $\mu$g/ml UTM-proteins reduced [$^3$H]thymidine incorporation by PHA-stimulated cells by 27% in the absence of heparin but only by 14% when heparin was present.

## Discussion

Present results confirm early findings (Segerson et al., 1984; Zhang and Miller, 1989; Skopets and Hansen, 1993) that UTM-proteins can reduce proliferation of PBL caused by PHA and extended these findings to PMA and antigen-induced proliferation. This lymphocyte-inhibitory effect is very likely due to UTM-proteins and not to contamination by other immunosuppressive proteins, because the proteins appear purified as determined by SDS-PAGE. Moreover,

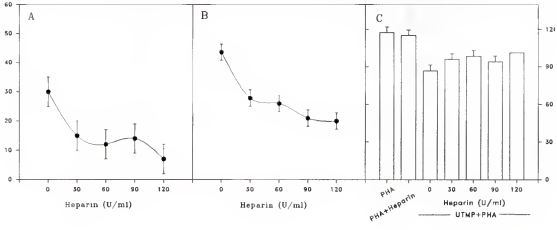


Figure 3-10. Effect of heparin on binding and lymphocyte inhibitory activity of UTM-proteins. Panel A. Inhibition of the binding of UTM-proteins to lymphocytes by heparin. Cells (1 x $10^6$ ) were incubated with 2 $\mu$g Bt-UTMP and various amounts of heparin in 100 $\mu$l staining buffer for 1 h on ice. After incubation, the cells were washed, incubated with streptavidin-PE. Binding was determined by FACScan. Results are least-squares means $\pm$ SEM from three ewes. Inhibition of binding by heparin was dose-dependent ($p < 0.01$). Panel B. Inhibition of Bt-UTMP binding to lymphocytes was caused by pretreatment with heparin. Cells (1 x $10^6$) were incubated with heparin in 100 $\mu$l staining buffer for 30 min on ice. After washing twice in staining buffer, cells were then incubated with 2 $\mu$g Bt-UTMP in 100 $\mu$l staining buffer for 1 h on ice. Following incubation with streptavidin-PE, binding was determined by FACScan. Results are least-squares means $\pm$ SEM from six ewes. There was dose-dependent inhibition caused by increasing amounts of heparin ($p < 0.001$). Panel C. Effect of heparin on inhibition of PHA-induced lymphocyte proliferation by UTM-proteins. PHA-treated PBL (1 x $10^6$) were cultured in a volume of 120 $\mu$l for 72 h in the presence of 500 $\mu$g/ml UTM-proteins and different amounts of heparin. Cell proliferation was measured by incorporation of [$^3$H]thymidine during last 12 h culture. Results are least-squares means $\pm$ SEM from seven ewes. Heparin reduced the inhibitory effects of UTM-proteins ($p < 0.001$).

lymphocyte-inhibitory activity of UTM-proteins was not neutralized by antibody to the immunosuppressive cytokine TGF-$\beta$(Skopets and Hansen, 1993). Interestingly, the purified sample of UTM-proteins contains lower molecular weight forms of UTM-proteins not identified by Coomassie Blue staining. Appearance of these proteins increases upon storage of uterine fluid (Hansen and Liu, 1994), suggesting that they are produced by proteolysis of UTM-proteins .

Concentrations of UTM-proteins required for inhibition of lymphocyte proliferation are higher than for cytokines that are immunosuppressants such as IFN-$\tau$ (Skopets et al., 1992) and TGF-$\beta$ (Stoeck et al., 1989). However, the inhibitory concentrations of UTM-proteins are physiological since similar or greater concentrations are present in the uterus during pregnancy (Moffatt et al., 1987). Also, another immunosuppressive serpin, $\alpha_1$ -AT, is inhibitory to lymphocytes at concentrations similar to these for UTM-proteins (Bata and Revillard, 1981; Hudig et al., 1981; Breit et al., 1985).

Exposure of PBL to PMA results in cell proliferation via enhancement of protein kinase C activity (Galron et al., 1994). This T cell proliferative process is independent of $Ca^{2+}$. PKC in turn causes phosphorylation of seryl and threonyl residues in transcription factors (Boulikas, 1995) and other proteins such as H1 histone (Mustelin et al., 1990). The fact that UTM-proteins blocked PMA-induced proliferation indicates that UTM-proteins affect signal transduction mediated by activation of PKC. Inhibition could directly affect at PKC activation or at a

downstream event. UTM-proteins did not interfere with IL-2 induced proliferation and actually increased expression of CD25. Results also indicated that UTM-proteins did not block lymphocyte proliferation through binding receptors for PHA and Con A.

Treatment with progesterone causes a decrease in the numbers of IEL in the uterus (Gottshall and Hensan, 1993). This effect could be a consequence of antiproliferative effects of UTM-proteins or because UTM-proteins block migration of lymphocytes into the uterus by causing down-regulation of homing receptors. UTM-proteins inhibited expression of $CD44^+$ $\gamma/\delta TCR^+$ lymphocytes. CD44 is a hyaluronic acid binding protein implicated in lymphocyte migration and homing (Lesley et al., 1993). In contrast, there was no effect of UTM-proteins on expression of two other cell adhesion molecules CD29 and L-selectin on $\gamma/\delta$ $TCR^+$ and $\gamma/\delta$ $TCR^+$ cells. Both adhesion molecules are also involved in lymphocyte migration (Li et al., 1993; Mackay et al., 1993).

The binding of UTM-proteins to lymphocytes could be an important step for inhibition of proliferation. Current findings indicate biotinylated UTM-proteins can bind to lymphocytes via a specific, dose-dependent and saturable manner. The receptor for UTM-proteins is probably distinct from the SECR that are expressed on neutrophils, monocytes (Pizzo et al., 1990; Perlmutter et al., 1990) and hepatocytes (Joslin et al., 1991) because complexes of antitrypsin-trypsin and thrombin-antithrombin III did not block binding of UTM-proteins to lymphocytes. It is possible

that UTM-proteins bind to serine proteinases on the PBL surface. Peripheral blood lymphocytes express serine proteinase such as elastase (Boistow et al., 1991) and DPPIV (Fleischer, 1994) on the surface. Inhibition of serine proteinase activity blocks lymphocyte proliferation (Tchorzewski et al., 1995). Dipeptidyl peptidase IV is analogous to CD26 and functions as a coreceptor that mediates signaling by the TCR-CD3 complex (Mittrucker et al., 1995).

UTM-proteins do not inhibit DPPIV enzymatic activity or lymphocyte costimulatory activity (Chapter V), but it is conceivable that they bind to cell surface CD26.

Heparin reduced the binding of UTM-proteins to lymphocytes and decreased inhibition of PHA-induced lymphocyte proliferation. Heparin may have exacted these effects in part by binding to soluble UTM-proteins since heparin binds to UTM-proteins (Zhang and Miller, 1989). However, heparin also reduced the binding of UTM-proteins to lymphocytes when added during preincubation of lymphocytes. Thus, heparin may modulate the binding of UTM-proteins to lymphocytes by reacting with cell surface proteins and blocking interaction between UTM-proteins and their receptors. Heparin has saturable and reversible binding sites on many types of cells through which this molecule can modulate cellular proliferation and differentiation (Barzu et al., 1985; Gallagher et al., 1986; Roberts et al., 1988; Kjellen et al., 1988; Bradbury et al., 1989).

In conclusion, the UTM-proteins suppress IL-2-independent proliferation of PBL but not inhibit expression of CD25 on $\gamma/\delta$ $TCR^+$ and $TCR^-$ lymphocytes. Binding

to lymphocytes is probably required to exert these inhibitory effects. UTM-proteins binding to lymphocytes is specific, dose-dependent and saturable process, and is blocked by heparin. UTM-proteins also down-regulate expression of CD44 on the surface of $\gamma/\delta$ $TCR^+$ cells. UTM-proteins may be good model proteins for studying how serpins can regulate lymphocyte functions.

# CHAPTER IV
# EFFECT OF THE PROGESTERONE-INDUCED SERPIN-LIKE PROTEINS OF THE SHEEP ENDOMETRIUM ON NATURAL-KILLER CELL ACTIVITY IN SHEEP AND MICE.

## Introduction

Natural killer cells are an important component of the natural immune system involved in immune surveillance against infectious diseases and autologous and allogeneic tumors (Trinchieri, 1989). Cell with characteristics of natural killer (NK) cells have been identified in the endometrium of mice (Parr et al., 1990), humans (King and Loke, 1991) and pigs (Croy et al., 1988). Of these, the granulated metrial gland (GMG) cell of the mouse uterus and large granular lymphocyte of the human endometrium have been studied most extensively. Both cells exhibit cytotoxic activity, surface markers, cytotoxins and proteases characteristic of NK cells (Croy et al., 1985; Stewart et al., 1988; King and Loke, 1990; Parr et al., 1990; Parr et al., 1991). To date, NK cells have not been identified in the sheep endometrium, but the major lymphocyte subpopulation in the endometrium is a $CD45R^+$ large granulated cells that have morphological properties similar to NK cells (Lee et al., 1988).

The functions of endometrial NK-like cells are unclear. It has been suggested that these cells could play an antiviral role or act as a source of cytokines to stimulate placental growth (King and Loke, 1990). Large granulated lymphocytes in the mouse metrial gland and human endometrium contain perforin and possess cytotoxic activity

towards trophoblast in vivo and in vitro (Stewart et al., 1984; Stewart et al., 1988; King and Loke 1990; Zheng et al., 1991). While the effect of polynucleotides on uterine NK-like cells is not known, activation of NK cells by polynucleotides can cause abortion in pregnant mice (Kinsky et al., 1990). It has been reported that peripheral blood lymphocytes from women with threatened pregnancies have significantly higher cytotoxic activity than women with normal pregnancies (Szekeres-Bartho et al., 1985).

One potential regulator of endometrial NK cell activity is progesterone. Administration of RU-486, a potent progesterone antagonist, augments natural killer cell activity in pregnant women (Hansen et al., 1992). In sheep, treatment of ovariectomized ewes with progesterone prolongs the survival of skin grafts in the uterine lumen (Hansen et al., 1986), reduces the number of $CD45R^+$ lymphocytes in the uterus (Gottshall and Hansen, 1992) and induces the appearance of lymphocyte-inhibitory molecules in uterine secretions (Stephenson and Hansen, 1990; Hansen and Skopets, 1992). The most abundant molecules exerting lymphocyte inhibitory activity in uterine fluid are the uterine milk proteins (UTM-proteins), which are a pair of related glycoproteins that comprise the major proteins in uterine fluid of progesterone-treated and pregnant ewes (Hansen et al., 1987; Ing et al., 1989; Stephenson and Hansne, 1990; Leslie and Hanen, 1991). The two proteins, having similar pI but slightly different molecular weights of 57,000 and 55,000 (Hansen et al., 1987), are members of the serpin superfamily of the serine protease inhibitors (Ing and Roberts,

1989), and are synthesized by epithelial cells of the uterine endometrium under influence of progesterone (Leslie and Hansen, 1991). The UTM-proteins interfere with activation of sheep lymphocytes induced by mitogen and mixed lymphocyte culture in vitro (Segerson et al., 1984; Zhang and Miller, 1989; Skopets and Hansen, 1993). Moreover, uterine fluid from progesterone-treated ewes suppresses [$^3$H]thymidine incorporation into mitogen-activated mouse spleen cells and inhibits T-cell dependent antibody production in vitro (Stephenson et al., 1991). These studies suggest that UTM-proteins play an important role in regulating immune responses in the uterus during pregnancy.

The inhibition of endometrial NK cell activity may be an important aspect of successful pregnancy because of the potential for actions directed against the conceptus. The aim of the present study was to test the hypothesis that UTM-proteins inhibit activity of NK cells. Specific objectives were to determine the effect of UTM-proteins on activity of sheep NK cells in vitro; establish the mouse as a model for study of effects of UTM-proteins on NK cell activity by testing the effect of the proteins on murine NK cells in vitro and Poly(I)·Poly(C) activation of NK cells in vivo; and evaluate whether UTM-proteins can reduce NK-cell mediated fetal loss.

## Materials and Methods

### Materials

RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), Histopaque-1077, penicillin,

streptomycin, L-glutamine, trypan blue, ovine serum albumin (OSA) and ovalbumin (Ova) were from Sigma (St. Louis, MO). For use in lymphocyte culture, RPMI-1640 was modified by addition of 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin, 2 mM supplemental L-glutamine and 10% (v/v) heat-inactivated bovine calf serum (Hyclone, Logan, UT). This medium is referred to as complete modified RPMI-1640 medium, whereas the medium without serum is referred to as incomplete modified medium. Sodium Chromium-51 (Sodium chromate in aqueous solution, 1 mCi/ml) and Poly(I)·Poly(C) were from Boehringer Mannheim (Indianapolis, IN). Bicinchonic acid (BCA) was from Pierce (Rockford, IL). The human chronic myelogenous leukemia cell line, K562, was obtained from Dr. Fuller W. Bazer of the Department of Animal Science, Texas A & M University and the YAC-1 mouse lymphoma cell line was from American Type Culture Collection (ATCC, Rockville, Maryland). Sephacryl S-200 HR and CM-Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ). Immersible-CX ultrafiltration devices were from Millipore (Bedford, Mass).

Animals

Healthy adult ewes, primarily of the Rambouillet breed, were used as blood donors and for collection of uterine fluid. Outbred mice of the ICR strain (35-42 d old) were from Charles River Laboratory (Wilmington, MA) and female C3H/HeJ mice (42-56 d old) were from Jackson Laboratory (Bar Harbor, Maine).

Preparation of UTM-Proteins

Uterine fluid was collected from the ligated horn of two unilaterally-pregnant ewes at d 136 or 139 of pregnancy as described (Bazer et al., 1979). Fluids were pooled together, centrifuged to remove precipitate (14476 X g for 30 min at 4 C), dialyzed (Mr cutoff=6000-8000) against 10 mM Tris-HCl (pH 8.2) and centrifuged at 14476 X g for another 30 min. Uterine fluid was loaded onto a column of CM-Sepharose (15 cm X 2.5 cm) pre-equilibrated in 10 mM Tris-HCl (pH 8.2). After washing the column in the same buffer, proteins binding to the column were eluted with 10 mM Tris-HCl (pH 8.2) containing 0.5 M NaCl. The basic protein fraction was concentrated with Millipore ultrafiltration devices and then further purified by gel filtration using a 94.4 X 1.5 cm column of Sephacryl S-200 HR and 10 mM Tris-HCl (pH 8.2) containing 0.33 M NaCl as the eluent. Purification of the proteins was confirmed by sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE). Typically, the purity was 90-95% with major impurities being lower molecular weight bands previously identified as breakdown products of UTM-proteins (Leslie et al., 1990). Purified UTM-proteins were dialyzed three times against DBPS and protein concentration was determined by the Lowry assay (Lowry et al., 1951) or BCA assay (Smith et al., 1985), using BSA as standard. Finally, the proteins were sterilized using a 0.22 $\mu$m filter and stored at -20 C in aliquots.

Preparation of Sheep Effector Cells

Peripheral blood lymphocytes were prepared from heparinized jugular blood of Rambouillet ewes using density gradient centrifugation on Histopaque-1077.

Briefly, the buffy coat layer of heparinized blood was placed on the top of an equal volume of Histopaque-1077 and centrifuged for 30 min at 400 X g. The cell layer at the interface was removed, washed twice with incomplete modified RPMI-1640, and resuspended to 1 x $10^7$ cells/ml in complete modified RPMI-1640. The cell suspension was placed in a 100 x 15 mm petri dish and incubated for 3 h at 37 C in 5% $CO_2$. The non-adherent cells were collected, washed twice in complete medium, adjusted to 1 x $10^7$ live effector cells/ml, and then incubated with UTM-proteins or Ovalbumin for cell lysis studies.

Preparation of Murine Splenocytes

The spleen was removed at sacrifice and placed in a 100 x 20 mm tissue culture dish containing 10 ml of incomplete modified RPMI-1640. After trimming the spleen, splenocytes were washed from the spleen by repeated injections of incomplete modified RPMI-1640. The cells were washed twice in incomplete modified RPMI-1640 and adjusted to 2.5 x $10^7$ cells/ml. Aliquots (2 ml) of cell suspension were placed on the top of an equal volume of Histopaque 1077 in a 17 x 100 mm culture tube and centrifuged at 400 x g for 30 min. Cells at the interface were collected, washed twice in modified RPMI-1640, resuspended to 1 x $10^7$ live cells/ml in complete modified medium and cultured for 1 h at 37 C and 5% $CO_2$ in a 100 x 15 mm petri dish. The cell suspension containing non-adherent cells was collected, centrifuged at 400 X g for 10 min to remove supernatant and resuspended in complete modified medium to a concentration of 5 x $10^6$ live effector cells/ml.

Labelling Target Cells

K562 and YAC-1 cell lines were continuously cultured in 10 ml complete modified RPMI-1640 in 25 $cm^2$ flasks at 37 C and 5% $CO_2$. The cells were subcultured twice weekly. Before labelling, cells were collected by centrifugation (300 X g for 10 min), washed three times in modified RMPI-1640, incubated with trypan blue to determine cell viability and resuspended to a concentration of 1 x $10^6$ live cells/ml. For labelling, 1 ml of the cell suspension was centrifuged at 300 X g for 10 min, 0.9 ml of supernatant were removed from the tube, 100 $\mu$l of $Na_2{}^{51}CrO_4$ (1 mCi/ml) were added and the tube was gently shaken. The cells were incubated at 37 C and 5% $CO_2$ for 90 min, washed three times in incomplete modified RPMI-1640, and then resuspended in 10 ml complete modified medium for the cytotoxicity assay.

Cytotoxicity Assay

The $^{51}Cr$-release assay was performed in 96-well V-shaped microplates (Dynatech Laboratory, Inc, Chantilly, VA) in a final volume of 0.2 ml per well. Each well contained 1 x $10^4$ labeled target cells and effector cells added in amounts to achieve effector:target cell ratios of 100:1, 50:1 and 25:1 in the sheep assay and 50:1, 25:1 and 12.5:1 in the mouse assay. After adding cells, plates were centrifuged at 150 X g for 5 min and then incubated at 37 C and 5% $CO_2$ for 4 h (mice) or 20 h (sheep). For the analysis of cytotoxicity, plates were again centrifuged at 300 X g for 10 min; 100 $\mu$l of each supernatant was collected and counted for radioactivity in a gamma scintillation counter. All assays were prepared in triplicate and results were

expressed as percent specific release using the formula: % specific release=(sample dpm - spontaneous dpm)/(maximum dpm - spontaneous dpm). Spontaneous release refers to the radioactivity in 100 $\mu$l of culture supernatant from culture containing 1 x $10^4$ labelled target cells in 200 $\mu$l medium. Maximum release refers to the radioactivity in 100 $\mu$l of supernatant from a mixture of 100 $\mu$l Triton X-100 and 100 $\mu$l medium containing 1 x $10^4$ labeled target cells.

Effect of UTM-Proteins on NK Cell Activity In Sheep

Sheep non-adherent lymphocytes (effector cells) were incubated in triplicate at 1 x $10^7$ live effector cells/ml with DPBS control, UTM-proteins (0.5 and 1.0 mg/ml) or Ova (1 mg/ml) for 20 h at 37 C and 5% $CO_2$. After washing cells three times in complete modified RPMI-1640, cell viability was examined by trypan blue exclusion and cell concentration was adjusted to 1 x $10^7$ live cells/ml in complete modified RPMI-1640. The cytotoxicity assay was performed using K562 target cells with an effector:target cell ratio of 100:1, 50:1 and 25:1 and a 20 h lysis time. Assays were replicated three times using lymphocytes from three separate donors.

Effect of UTM-Proteins on Activity of Murine NK Cells

Murine splenocytes were incubated in triplicate at 1 x $10^7$ cells/ml with UTM-proteins (final concentrations=0, 0.1, 0.5 and 1 mg/ml) or OSA (1 mg/ml) for 4 h at 37 C and 5% $CO_2$. After incubation, cells were washed three times in complete modified RPMI-1640, cell viability was examined by trypan blue and cell concentration was adjusted to 5 x $10^6$ live cells/ml for a 4 h $^{51}Cr$ release assay using

YAC-1 cells as targets.

Effect of UTM-Proteins on NK Cell Activation Induced by Poly(I)·Poly(C)

The design was a 2 x 2 factorial with main effects of Poly(I)·Poly(C) (20 $\mu$g or DPBS vehicle) and protein treatment (UTM-proteins or DPBS vehicle). Additionally, a fifth group was used that received OSA (control protein) without Poly(I)·Poly(C). The dose of Poly(I)·Poly(C) was chosen because this amount was able to increase fetal loss in mice[Kinsky et al., 1990). Female mice of the ICR outbred strain (35-47 days old) were used (3-4 mice group). On the morning of d 0, mice were injected, i.p., with 1.2 ml of DPBS containing appropriate treatments (0 or 20 $\mu$g Poly(I)·Poly(C); no protein, 3 mg UTM-proteins or 3 mg OSA). Another injection of 1.0 ml DPBS, 3 mg UTM-proteins in 1 ml DPBS or 3 mg OSA in 1 ml DPBS was given on the afternoon of d 0. On d 2, all mice were sacrificed and spleens were removed. Non-adherent splenocytes were prepared using previously described procedures and used to determine NK activity with YAC-1 target cells in a 4-h $^{51}$Cr release assay.

Effect of UTM-Proteins on Embryonic Degeneration Induced by Poly(I)·Poly(C)

The experimental design was a 2 x 3 factorial with main effects of Poly(I)·Poly(C) (20 $\mu$g or DPBS vehicle) and protein treatment (UTM-proteins, OSA or DPBS vehicle). Females of the ICR outbred strain mice (35-47 days old) were mated to ICR outbred strain males; the day of vaginal plug was designated as d 0 of gestation. Bred females were assigned randomly to six treatments of 5-10 mice per

treatment. At d 3 or 4 of pregnancy, two consecutive i.p. injections were administered 24 h apart. The first injection was 1 ml UTM-proteins (3 mg), OSA (3 mg) or DPBS vehicle, and for some groups, 20 $\mu$g Poly(I)·Poly(C). On the following day, a second injection of 1 ml UTM-proteins (3 mg), OSA (3 mg) or DPBS vehicle was administered. On d 13 or 14 of pregnancy, mice were sacrificed, and living fetuses, early degenerative conceptuses and late degenerative/stunted fetuses were counted. Early degeneration was considered as an implantation site without a visible embryo. A fetus was considered as a late degenerative/stunted fetus if its weight was very small compared to other normal litter mates or if obvious morphological signs of mummification or degeneration were present.

Statistical Analysis

All data were analyzed by least squares analysis of variance using the General Models Program of the Statistical Analysis System (SAS, 1985). Data concerning effects of UTM-proteins on sheep NK cell activity were analyzed with a model including main effects of donor, ratio of effector:target cell ratio and treatments. For analysis of inhibition of murine NK cell activity by UTM-proteins, data were analyzed using a model that included main effects of donor, concentration of UTM-proteins and effector:target cell ratio. A second analysis was performed to evaluate the effect of OSA. To determine the effect of UTM-proteins on Poly(I)·Poly(C) induced NK activity, data were analyzed with main effects of treatment and effector:target cell ratio. Additionally, data were analyzed as a 2 x 2 factorial after removing the OSA

group. The statistical model included main effects of Poly(I)·Poly(C), protein treatment and effector:target cell ratio. Effects of UTM-proteins on Poly(I)·Poly(C)-induced abortion were analyzed using a similar model. For all statistical analyses, donor was considered a random effect and other main effects were considered fixed. Additionally, mathematical models for statistical analysis were constructed to include all interactions. When appropriate, preplanned orthogonal contrasts were used to partition treatment effects.

## Results

### Effect of UTM-proteins on NK Cell Activity in Sheep and Mice

Preincubation of non-adherent sheep mononuclear cells for 20 h did not reduce their viability which averaged 94 $\pm$ 1% (mean $\pm$ between animal SEM) for cells cultured with vehicle, 95 $\pm$ 0% for cells cultured with 1 mg/ml Ova, 93 $\pm$ 1% for cells incubated with 0.5 mg/ml UTM-proteins and 93 $\pm$ 2% for cells cultured with 1 mg/ml UTM-proteins. Preincubation with both concentrations of UTM-proteins did, however, significantly reduce ability of the cells to lyse $^{51}$Cr-labelled K562 target cells (Figure 4-1). Preincubation with 1 mg/ml Ova tended to reduce lysis at the 100:1 effector:target ratio only (ratio x treatment; $P < 0.03$) but to a much lesser degree than for 1 mg/ml UTM-proteins.

Incubation of non-adherent mouse splenocytes with UTM-proteins also had no

significant effect on their viability. Viability at the end of 4 h incubation was 95 $\pm$ 0% for control cells, 95 $\pm$ 0% for cells cultured with OSA and 92 $\pm$ 4%, 87 $\pm$ 2%

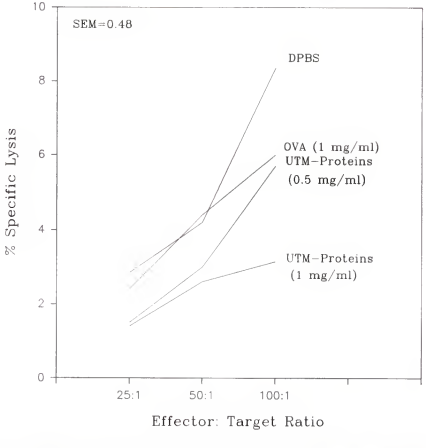


Figure 4-1. Inhibition of NK cell activity of sheep non-adherent peripheral blood lymphocytes by preincubation with UTM-proteins for 20 h. Results are expressed as percentage of maximum lysis of K562 cells in a 20 h $^{51}Cr$ release assay. Value represents least-square means. Lysis was affected by treatment ($P<0.03$), effector:target cell ratio ($P<0.01$) and the ratio x treatment interaction ($P<0.03$). Using orthogonal contrasts, control (DPBS) + ovalbumin (OVA) > UTM-proteins (UTMP) ($P<0.001$) and controls tended to differ from ovalbumin ($P<0.07$).

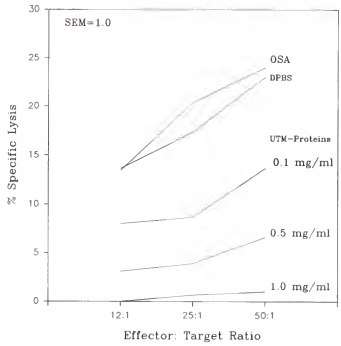


Figure 4-2. Inhibition of NK activity of non-adherent murine splenocytes by 4 h preincubation with UTM-proteins. The cytotoxicity of NK cells against YAC-1 cells was determined in a 4 h $^{51}Cr$ release assay. Lysis (expressed as percent of maximum release) was affected by concentration of UTM-proteins ($P<0.01$), effector cell:target cell ratio ($P<0.01$) and the ratio x concentration interaction ($P<0.05$). OSA had no effect on lysis, but there was a ratio x OSA interaction ($P<0.07$).

and 86 $\pm$ 2% for cells cultured with 0.1, 0.5 and 1.0 mg/ml UTM-proteins (mean $\pm$ between-animal SEM). At all effector:target ratios tested, UTM-proteins reduced ($P<0.01$) ability of NK cells to lyse target cells (Figure 4-2). For example, at a ratio of 50:1, percent lysis of target cells by control NK cells was 23% (least-squares mean) vs. 13.7, 6.6 and 1.0% for cells cultured with 0.1, 0.5 and 1.0 mg/ml UTM-proteins. Incubation with OSA did not affect NK cell activity.

Effect of UTM-proteins on Activation of NK Cells by Poly(I)·Poly(C)

Overall, UTM-proteins affected NK activity ($P<0.01$) and there was no interaction with Poly(I)·Poly(C) (Table 4-1). For mice not treated with Poly(I)·Poly(C), treatment with UTM-proteins reduced basal lytic ability of NK cells. Injection of Poly(I)·Poly(C) increased the lytic activity of NK cells for control mice and those treated with UTM-proteins but lysis was lower for cells from animals treated with UTM-proteins.

Table 4-1. Effect of UTM-proteins on Poly(I)·Poly(C)-induced activation of NK cells in mice: percent lysis of YAC-1 target cells.

| | Effector:target ratio[a] | | |
|---|---|---|---|
| Treatment[b] | 50:1 | 25:1 | 12.5:1 |
| Vehicle | 15.5 | 9.7 | 4.6 |
| Ovine serum albumin | 16.6 | 14.5 | 12.3 |
| UTM-proteins | 4.5 | 3.4 | 2.2 |
| Poly(I)·Poly(C) | 34.5 | 26.4 | 18.9 |
| Poly(I)·Poly(C) + UTM-proteins | 18.2 | 14.1 | 9.9 |

[a]Results are least-squares means of results from 3-4 mice per group. Pooled SEM = 0.93.

[b]Lysis was affected by ratio ($P<0.001$) and treatment ($P<0.001$). When data were analyzed as a factorial experiment after excluding the OSA treatment, lysis was affected by ratio ($P<0.001$), Poly(I)·Poly(C) ($P<0.001$) and treatment ($P<0.01$).

Effect of UTM-proteins on Fetal Loss Induced by Poly(I)·Poly(C)

Results are shown in Table 4-2. Injection of Poly(I)·Poly(C) increased the percentage of fetuses undergoing late degeneration/stunting ($P<0.05$). There was also a nonsignificant tendency for Poly(I)·Poly(C) to increase the percent of fetuses undergoing early degeneration. Injection of UTM-proteins reduced the effect of

Poly(I)·Poly(C) on percentage of early (not significant), late ($P=0.09$) and total degenerations ($P=0.06$). For example, injection of Poly(I)·Poly(C) increased the percent fetuses undergoing early degeneration in vehicle-treated mice from 4.5 to 7.1% and the percent undergoing late degeneration from 0.0 to 5.0%. However, when mice received UTM-proteins, there was no increase in the percent fetuses undergoing early (3.1 vs 2.4%) or late degeneration (1.0% vs 0.8%). Injection with OSA did not affect percent of fetuses undergoing degeneration.

Table 4-2. Effect of UTM-proteins on Poly(I)·Poly(C)-induced embryonic degeneration in mice[a].

| Poly(I)·Poly(C) | Treatment | n | No.of implantations | Degenerated conceptuses (%) Early | Late | Total |
|---|---|---|---|---|---|---|
| 0.0 | Vehicle | 9 | 13.0 | 4.5 | 0.0 | 4.5 |
| 0 | OSA | 5 | 11.0 | 3.6 | 0.0 | 3.5 |
| 0 | UTM-proteins | 10 | 12.4 | 3.1 | 1.0 | 3.8 |
| 20 $\mu$g | Vehicle | 9 | 13.1 | 7.1 | 5.0 | 12.1 |
| 20 $\mu$g | OSA | 6 | 11.3 | 5.8 | 4.2 | 10.0 |
| 20 $\mu$g | UTM-proteins | 10 | 12.1 | 2.4 | 0.8 | 3.3 |
| Poly(I)·Poly(C) | | | N.S. | N.S. | 0.02 | 0.04 |
| UTM-proteins vs others | | | N.S. | N.S. | N.S. | 0.08 |
| OSA vs vehicle | | | 0.01 | N.S | N.S. | N.S. |
| Poly(I)·Poly(C) x (UTM-proteins vs others) | | | N.S. | N.S. | 0.09 | 0.06 |
| Poly(I)·Poly(C) x (OSA vs vehicle) | | | N.S. | N.S. | N.S. | N.S. |
| SEM | | | 0.5 | 1.9 | 0.6 | 2.6 |

[a]Results are least-squares means. N.S. represents nonsignificant ($P > 0.10$).

## Discussion

In the present study, UTM-proteins reduced cytotoxic activity of sheep and mouse NK cells. In addition, injection of UTM-proteins into mice inhibited basal splenocyte NK-cell activity in vivo. While not preventing stimulatory effects of Poly(I)·Poly(C) on splenocyte cytotoxic activity, such activity remained lower for UTM-protein treated mice after Poly(I)·Poly(C) treatment. The inhibitory effects of UTM-proteins in vivo reflect either inhibition of NK-cell activation or alteration of movement of NK cells through the spleen. The former explanation is more likely because few peripheral NK cells enter the spleen (Warner and Nennert, 1982). UTM-proteins also blocked fetal loss induced by Poly(I)·Poly(C). These results provide good evidence that UTM-proteins can suppress the cytolytic activity of NK cells and, together with earlier findings that UTM-proteins can block proliferation of T lymphocytes (Segerson et al., 1984; Zhang and Miller, 1989; Skopets and Hanen, 1993), suggest that the proteins may be important regulators of immune cells during pregnancy.

It is also feasible that the UTM-proteins are responsible for mediating effects of progesterone on intrauterine skin graft survival (Hansen et al., 1986) and changes in endometrial lymphocyte populations (Gottshall and Hansen, 1992). The concept that progesterone can regulate uterine immune function indirectly by inducing the synthesis of endometrial proteins is likely to be relevant for other species. Progesterone induces lymphocyte-inhibitory activity in uterine fluid of cows (Lander

et al., 1990) and explants of human endometrium (Wang et al., 1988). Additionally, proteins similar to UTM-proteins have been found in the cow (Ing and Roberts, 1989; Leslie and Hansen, 1991) and pig (Malathy and Imakawa, 1989) and it is possible that these proteins may also be immunoregulatory during pregnancy. Placental protein 14, a progesterone induced protein of the human endometrium, also suppresses NK-cell activity (Okamoto et al., 1991).

The fact that UTM-proteins inhibit murine NK cells and that ovine uterine fluid inhibits murine lymphocyte proliferation in vitro (Stephenson et al., 1991) indicates there is a degree of similarity between sheep and mice in regulation of immune functions. Accordingly, the murine system can provide a useful model for evaluating effects of UTM-proteins on immune function, especially for in vivo studies, because amounts of UTM-proteins required are greatly reduced as compared to comparable studies in the ewe. Additionally, optimum conditions for ovine NK cells have not been established: the low degree of lysis of K562 cells by NK cells from Rambouillet sheep in the present study was similar to that for effector cells from this breed of sheep in another study (Tuo et al., 1993). In contrast to results with sheep lymphocytes, murine NK cells are highly active, perhaps because target cells for this species have been better described for this species or cells survive purification better than for the sheep. Perhaps killing of target cells in the sheep system was caused by cells distinct from classical NK-cell lineages in other species.

In mice, infiltration of NK cells into the decidua was associated with

spontaneous abortion (Gendron and Baines, 1988) and therefore activation of NK-like cells during pregnancy may lead to destruction of the placenta. Kinsky (Kinsky et al., 1990) found that injection of Poly(I)·Poly(C) and Poly(I)·Poly(C12U) increased embryonic loss through activation of NK cells. It is likely, therefore, that UTM-proteins reduced the effectiveness of Poly(I)·Poly(C) in causing abortion either because it blocked activation of NK cells (of intrauterine or extrauterine origin) or GMG cells or because UTM-proteins prevented the effects of Poly(I)·Poly(C) on release of interferon (Djeu et al., 1979; Youn et al., 1983).

UTM-proteins are members of the serine protease inhibitor serpin family (Ing and Roberts, 1989). Interestingly, UTM-proteins, which do not inhibit trypsin (Hansen et al., 1987), share two properties with the serpin $\alpha$1-antitrypsin; binding to IgA (Hansen and Newton, 1988) and inhibition of NK cell and T-cell function [Hudig et al., 1981; Breit et al., 1983). It has been shown that dipeptidyl protease IV, a serine protease, is involved in the activation and function of T-cells (Bristol et al., 1992) and proteases play an important role in the NK cell-mediated cytolysis of target cells (Ewoldt et al., 1992). Therefore, it is possible that UTM-proteins regulate lymphocyte activity through interference of proteases required for lymphocyte function. Some members of the serpin superfamily also function as binding proteins for small, poorly water-soluble molecules (Pemberton et al., 1988) and it remains possible that inhibitory activity associated with UTM-proteins is actually due to an associated ligand for the proteins. Lymphocyte-inhibitory activity in uterine fluid from pregnant ewes

was not associated with a lipid-soluble fraction and could be reduced by protease treatment (Stephenson et al., 1988). It remains likely, therefore, that UTM-proteins were responsible for the inhibition of NK-cell activity seen in the present studies. These findings strengthen the idea that these proteins play an important role in modulating function of endometrial lymphocytes during pregnancy so as to affect the survival of the conceptus.

# CHAPTER V
# PROGESTERONE-INDUCED SECRETION OF DIPEPTIDYL PEPTIDASE IV (CD26) BY THE UTERINE ENDOMETRIUM OF THE EWE AND COW THAT COSTIMULATES LYMPHOCYTE PROLIFERATION

## Introduction

Dipeptidyl peptidase IV (DPPIV, E.C.3.4.14.5) is a serine protease that preferentially hydrolyses a peptide bond on the carboxyl side of a penultimate proline (Walter et al., 1980). The enzyme is a type II transmembrane glycoprotein that is widely distributed throughout mammalian cells and tissues, being most abundant on the brush borders of epithelial cells (hepatocytes, enterocytes, pancreatic duct cells and salivary duct cells), T lymphocytes, and placental cells (Kojma et al., 1980; Puschel et al., 1982; Fox et al., 1984; Hafter et al., 1985; Fleischer, 1994). Dipeptidyl peptidase IV also has been found on the apical plasma membrane of rabbit uterine epithelium by histochemical staining (Classen et al., 1987) and can be detected in serum (Sanda et al., 1989). In T lymphocytes, DPPIV is recognized to be cluster differentiation antigen (CD) 26 (Matern et al., 1991; Marguet et al., 1992) and to play an important role in coactivation of T lymphocyte proliferation in vitro and immune responses in vivo (Morimoto et al., 1989; Flentke et al., 1991; Torimoto et al., 1991). DPPIV has also been implicated in CD16-mediated lysis by NK cell activation (Madueno et al., 1993). The enzymatic activity of DPPIV is required for its role in T cell functions (Schon et al., 1989; Kubota et al., 1992; Tanaka et al., 1993). The

precise roles of DPPIV in other tissues are unclear. One possibility is that the enzyme is involved in regulation of cellular and extracellular matrix interactions: DPPIV has affinity for collagen and fibronectin and has been suggested to participate in interactions between cells and extracellular matrix to affect cell migration and other functions (Hanski et al., 1985; McCaughan et al., 1990; Fleischer, 1994). Additionally, many hormones and cytokines have the N-terminal sequence X-Pro and are potential substrates of DPPIV (Fleischer, 1994).

In sheep, the uterine endometrium contains subclasses of lymphocytes which are decreased by treatment of progesterone (Gottshall and Hansen, 1992) and increased during late pregnancy (Lee et al., 1992). These changes likely result from endometrial regulatory factors controlling lymphocyte proliferation or recruitment. One such factor is a pair of progesterone-induced endometrial secretory glycoproteins (Leslie and Hansen, 1991) called the uterine milk proteins (UTM-proteins) (Leslie and Hansen, 1991) which can inhibit cellular immune responses (Skopets and Hansen, 1993; Liu and Hansen, 1993). Dipeptidyl peptidase IV of endometrial origin could participate in this local regulation of lymphocyte function. To examine the possible role of DPPIV in uterine function, experiments were conducted to; 1) test whether DPPIV in present in uterine secretions of sheep and cattle; 2) determine regulation of uterine DPPIV by pregnancy and progesterone; 3) characterize biochemical properties of endometrial DPPIV; 4) identify the distribution of DPPIV-positive cells in sheep endometrium; 5) determine the effect of DPPIV on proliferation of mitogen-stimulated

lymphocytes; and 6) test whether UTM-proteins inhibit actions of DPPIV on lymphocyte proliferation.

## Materials and Methods

### Materials

Bicinchonic acid (BCA) was purchased from Pierce (Rockford, IL). The $NH_2$-$(CH_2)_6$-$NH_2$-Sepharose 4B, CM-Sepharose, Sephacryl S-200 HR and concanavalin A-Sepharose 4B were obtained from Pharmacia (Piscataway, NJ). issue Culture Medium 199 (TCM-199), Dulbecco's phosphate-buffer saline (DPBS), fetal calf serum, Histopaque 1077, phytohemagglutinin-L (PHA), ovine serum albumin (OSA), methyl-α-D-glucoside, normal mouse ascites fluid, progesterone, *p*-nitroanilide, glycyl-proline, Gly-pro-*p*-nitroanilide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were from Sigma (St Louis, MO). Ampholytes (Iso-Lyte 4-8) were from ICN Biomedicals (Costa Mesa, CA). Centricon P-100 microconcentration devices were from Amicon (Danvers, MA) and Immobilon membranes were from Millipore (Bedford, MA). 2-N-Morpholino ethanesulfonic acid sodium salt (MES) was from Research Organics (Cleveland, Ohio). Anti-Tal monoclonal antibody against human CD26 was from Coulter Immunology (Hialeah, FL) and the peroxidase immunohistochemistry staining kit was from Biomeda (Foster, CA). Microtiter plates were purchased from Becton Dickinson (Oxnard, CA). [$^3$H-methyl]thymidine (specific activity= 5 Ci/mmol) was from Amersham (Arlington Heights, IL).

### DPPIV in Uterine Fluids of Unilaterally-Pregnant Ewes

Uterine fluids were collected from the ligated uterine horn of unilaterally-pregnant Rambouillet ewes at day 60 (n=3), 100 (n=3) and 140 of gestation (n=3). Unilateral pregnancies were prepared by placing a ligation around one uterine horn, removing the ovary and breeding ewes by natural mating (Bazer et al., 1979). Uterine fluid was collected by aspiration using a syringe and 18 gauge needle after exposure of the uterus by laparotomy or removal of the uterus at slaughter. Fluids were centrifuged at 10,000 x g for 10 min and stored at -20 C until further analysis.

Induction of DPPIV by Progesterone

Details of treatment of these animals have been reported previously (Leslie and Hansen, 1991). Ovariectomized Suffolk, Rambouillet or Rambouillet crossbred ewes were treated daily with subcutaneous injections of 2 ml corn oil vehicle for 10 days (n=3) or 100 mg progesterone in 2 ml corn oil for 30 (n=3) or 60 (n=3) days (Leslie and Hansen, 1991). Ovariectomized Holstein cows received daily subcutaneous injections of 15 ml corn oil vehicle for 12 days or 750 mg progesterone in 15 ml corn oil for 12 (n=3) or 30 (n=3) days. Animals were slaughtered on the morning following the last injection. Immediately following exsanguination, reproductive tracts were removed and taken to the laboratory for further processing. After clamping the cervix, tracts were flushed with 20 ml sterile saline. Fluid was centrifuged at 400 x g for 20 min, divided into aliquot of 1 ml, and frozen at -20 C until further analysis.

Enzyme Assay

Enzymatic activity of DPPIV was measured using a modification of the method of Nagatsu et al. (Nagatsu et al., 1976). Briefly, 100 $\mu$l sample and 100 $\mu$l substrate (2 mM Gly-pro-*p*-nitroanilide in 0.01 M Tris-HCl, pH 8.2) were placed in wells of 96-well flat-bottom microtiter plates. The assay was carried out at room temperature. Absorbance was determined at wavelengths of 405 nm and 570 nm immediately following addition of the substrate and again after 15 min incubation at room temperature through use of a Bio-Tek Model EL 309 microtiter plate reader (Wincoski, VT). Amounts of formed product were determined from a standard curve of *p*-nitroaniline. One enzyme unit was defined as the amount of enzyme catalyzing the formation of 1 $\mu$mol of *p*-nitroaniline per min at pH 8.2 and room temperature.

Preparation of Gly-pro-NH-$(CH_2)_6$-NH-Sepharose 4B

The column was prepared by adding 45 mg glycyl-proline and 60 mg 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide to 12 ml wet $NH_2$ $(CH_2)_6$-NH-Sepharose 4B at pH 4.5 (MacNair et al., 1979). The reaction proceeded at 10 C overnight on a tube turner. Unbound peptide was then washed from the matrix with 0.02 M acetate buffer (pH 6.0). The concentration of coupled protein was 2.85 mg/ml gel.

Purification of DPPIV

Uterine fluids from two unilaterally pregnant ewes (d 140 of pregnancy) were thawed, pooled, centrifuged (14,476 x g for 30 min at 4 C) to remove precipitate, dialyzed ($M_r$ cutoff=6000-8000) against 10 mM Tris-HCl, pH 8.2, and centrifuged at 14476 x g for 30 min. Aliquots (10 ml) of the supernatant fraction of dialyzed

uterine fluid were loaded onto a column of CM-Sepharose (15 cm x 2.5 cm) pre-equilibrated in 10 mM Tris-HCl, pH 8.2, and washed with the same buffer. The proteins not binding to the column were collected and pH was adjusted to 7.4 with 0.1 N HCl; Proteins were then loaded onto a column of concanavalin A-Sepharose (0.5 x 7 cm) pre-equilibrated with 0.01 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, 0.7 mg/l pepstatin and 0.5 mg/l leupeptin. Bound proteins were eluted with the same buffer containing 0.5 M methyl-α-D-glucoside. The pooled eluant was concentrated using Millipore ultrafiltration devices and further purified by gel filtration using a 94.4 X 1.5 cm column of Sephacryl S-200 HR with 0.01 M Tris-HCl, pH 8.2, containing 0.33 M NaCl as column buffer. Pooled fractions containing DPPIV were dialyzed against 0.01 M Tris-HCl, pH 8.2 and then further purified by chromatography on a 1.5 x 7 cm column of gly-pro-NH-$(CH_2)_6$-NH-Sepharose 4B pre-equilibrated with 0.01 M Tris-HCl, pH 8.2. The column was washed with the same buffer, and bound proteins were eluted with a linear gradient of NaCl (from 0 to 0.2 M). The final pooled enzyme solution was concentrated using either a Centricon 50 microconcentration device to remove proteins less than 50 kDa (for two dimensional gel electrophoresis) or a Centricon 100 device followed by dialysis against DPBS (for in vitro lymphocyte proliferation assays). All purification steps were carried out at 10 C; 0.02% (wt/vol) $NaN_3$ was included in all buffers except DPBS.

Purification of UTM-Proteins

Uterine fluids were pooled from separate ewes, centrifuged to remove

precipitate (14476 x g for 30 min at 4 C), dialyzed ($M_r$ cutoff = 6000-8000) against 10 mM Tris-HCl, pH 8.2, and centrifuged at 14476 x g for another 30 min. Uterine fluid was loaded onto a column of CM-Sepharose (15 cm x 2.5 cm) pre-equilibrated in 10 mM Tris-HCl, pH 8.2. The basic protein fraction was eluted from the column using 10 mM Tris-HCl, pH 8.2, containing 0.5 M NaCl and then further achieved by gel filtration using a 94.4 x 1.5 cm column of Sephacryl S-200 HR and 10 mM Tris-HCl (pH 8.2) containing 0.33 M NaCl as the eluent. Purification was determined by one dimensional electrophoresis. The purity was greater than 90%, with the remaining protein being lower molecular-weight breakdown products of UTM-proteins. Purified UTM-proteins were dialyzed three times against DPBS, sterile-filtered using a 0.22-$\mu$m filter and stored in aliquots at -20 C.

Polyacrylamide-Gel Electrophoresis

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions by the method of Laemmli (Laemmli, 1970) using 7.5% polyacrylamide gels. Gels were stained with 0.125% (wt/vol) Coomassie Blue. Two-dimensional SDS-PAGE was carried out as described by Roberts et al. (1984). Separation of the proteins in the first dimension was performed using ampholytes with a pI range of 4.0 to 8.0 and separation in the second dimension was under reducing conditions in 7.5 % (w/v) polyacrylamide gels. Proteins were stained with 0.125% Coomassie blue.

Protein Concentration Determination

Protein content of samples was determined using the bicinchonic acid modification of the Lowry procedure with bovine serum albumin (BSA) as a standard (Smith et al., 1985).

Characterization of DPPIV Enzymatic Activity

The pH optimum for DPPIV enzymatic activity was determined using a universal buffer containing 0.05 M citrate-phosphate-borate/HCl. Crude uterine fluid (100 $\mu$l) was incubated with 100 $\mu$l 2 mM Gly-pro-*p*-nitroanilide in buffer at various pH at room temperature; absorbance was read at 405 and 570 nm at times 0 and 15 min following addition of substrate. The $K_m$ was determined in 10 mM Tris-HCl at pH 8.2 by reacting various concentrations of Gly-pro-*p*-nitroanilide with partially purified DPPIV at room temperature; $K_m$ was calculated by Lineweaver-Burk plot. Inhibition of DPPIV enzymatic activity by UTM-proteins was determined by incubation of partially-purified DPPIV and substrate with various concentrations of UTM-proteins in 10 mM Tris-HCl containing 0.2 M NaCl, pH 8.2, at room temperature for 5, 15 and 30 min.

$NH_2$-Terminal Amino Acid Sequencing

A sample of DPPIV (partially purified by cation exchange, lectin, gel filtration and affinity chromatography using Gly-pro NH-$(CH_2)_6$-NH-Sepharose 4B) was subjected to two-dimensional gel electrophoresis under reducing conditions. Proteins were then electrophoretically transferred onto Immobilon membrane in 10 mM MES, pH 6.0, containing 20% (vol/vol) methanol. The transfer at 200 mA proceeded

overnight at room temperature. Transferred proteins were visualized by staining with 0.1% (wt/vol) Coomassie Blue R-250. A protein spot at 107 kDa was excised for microsequencing analysis. Automated Edman degradation of proteins was performed using an Applied Biosystems sequencer (model 470A) equipped with an on-line HPLC for phenylthiohydantoin amino acid identification.

Immunohistochemistry

Intercaruncular endometrium from the pregnant horn of unilaterally-pregnant ewes at days 60 (n=2) and 140 (n=3) of pregnancy and from ovariectomized ewes treated with corn oil (n=2) or progesterone for 30 d (n=2) or 60 d (n=2) were fixed in Bouin's fixative, dehydrated and embedded as described (Leslie and Hasnen, 1991). Rehydrated deparaffinized tissue sections (5 $\mu$m) were incubated with monoclonal antibody to human CD26 (Fox et al., 1984) diluted 1:200 with DPBS for 2 h at room temperature and then rinsed with PBS for 3 minutes. Tissue sections were incubated with peroxidase-conjugated second antibody to mouse IgG (whole molecule) for 30 min, rinsed with PBS for 3 min, and then incubated with substrate according to manufacture's instructions. Tissue sections were counterstained with hematoxylin and covered with a cover slide using glycerol-gelatin. A negative control for non-specific binding was done by using normal mouse ascites fluid in place of primary antibody.

Effect of DPPIV on PHA Stimulated-Lymphocyte Proliferation

Sheep peripheral blood lymphocytes (n=4) were obtained by centrifugation using Histopaque-1077 (Low and Hansen, 1988). Lymphocytes were suspended to

1 x $10^6$/ml in TCM 199 containing 5% (vol/vol) horse serum, 1 x $10^{-4}$M pyruvate and 5 x $10^{-5}$ M BME. Aliquots (100 $\mu$l) of cells were placed in wells of a 96-well microplate in a final volume of 110 $\mu$l containing PHA (2.5 $\mu$g/ml) and various amounts of DPPIV (or DPBS control) for 72 h at 37 C and 5% $CO_2$. After this time, 0.1 $\mu$Ci [$^3$H] thymidine was added in 10 $\mu$l medium. Cells were harvested 12 h later as previously described (Low and Hansen, 1988) and radioactivity incorporated into cells was determined by scintillation spectrometry.

Inhibition of Action of DPPIV on Lymphocyte Proliferation by Uterine Milk Proteins

Purified sheep peripheral blood lymphocytes from 2 ewes were cultured separately at 1 x $10^5$ cells/100 $\mu$l modified TCM-199 with PHA (0 or 2.5 $\mu$g/ml) and DPPIV (0 or 2.3 $\mu$g/ml), OSA (500 $\mu$g/ml) or UTM-proteins (100, 200, 400 or 500 $\mu$g/ml) for 72 hr at 37 C and 5% $CO_2$. After 72 h, 0.1 $\mu$Ci [$^3$H] thymidine was added. Cells were harvested 12 h later and radioactivity incorporated into cells was measured by scintillation spectrometry.

Statistical Analysis

Data were analyzed by least-squares analysis of variance using the GLM procedure of the Statistical Analysis System (SAS, 1989). Data were sometimes log-transformed before analysis to reduce heterogeneity of variance. Animal was considered as a random effect and other main effects were considered as fixed. In some analyses, orthogonal contrasts were used to separate treatment effects into individual degree-of-freedom comparisons. For example, variance associated with the

effect of progesterone treatment on induction of DPPIV in sheep was partitioned into effects of 1) vehicle vs progesterone-treated ewes and 2) progesterone treatment for 10 days vs progesterone treatment for 30 days. Contrasts were made using orthogonal polynomials to determine whether dose response curves describing effects of DPPIV and UTM-proteins were linear, quadratic, cubic, quartic or quintic in nature.

## Result

### Regulation of DPPIV Accumulation in Uterine Secretions

In unilaterally pregnant ewes, enzymatic activity of DPPIV in uterine secretions of the ligated uterine horn increased as pregnancy progressed ($P < 0.01$). This was true for both enzyme concentration and total activity (Table 5-1). From day 60-140 of pregnancy, there was a 44-fold increase in total DPPIV in uterine fluid. In ovariectomized ewes and cows, activity of DPPIV in uterine flushings was increased ($P < 0.001$) by treatment with progesterone in a time-dependent manner (Table 5-2). Activity of DPPIV was very low in ovariectomized animals not receiving progesterone. Treatment with progesterone caused an approximately 10 and 385 fold increase after 30 and 60 d of treatment in ewes and a 1.8 and 18.8 fold increase after 12 and 30 d of treatment in cows.

### Purification of DPPIV

Dipeptidyl peptidase in crude uterine fluid did not bind to CM-Sepharose at pH 8.2. This step allowed separation of DPPIV from the major

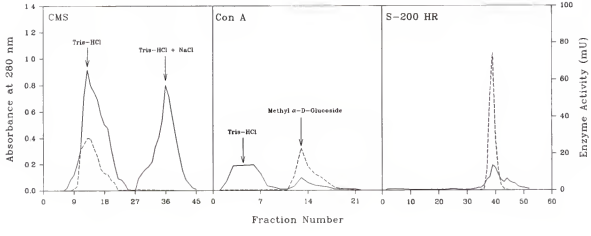


Figure 5-1 DPPIV eluted in the unbound fraction when dialyzed uterine fluid was placed on a column of CM-Sepharose in 0.01 M Tris-HCl (pH 8.2) (left panel). Subsequent chromatography of the active fractions using Con A-Sepharose indicated that all activity bound to the column and could be eluted with buffer containing 0.5 M methyl-α-D-glucose (middle panel). Further chromatography by gel filtration using Sephacryl S-200 HR with a buffer of 0.01 M Tris-HCl (pH 8.2) containing 0.33 M NaCl resulted in a single peak of Mr=140,000. Absorbance was measured at 280 nm (__). Enzyme activity was determined using Gly-p-*p*-nitroanilide as substrate (.....).

Table 5-1. Activity of dipeptidyl peptidase IV in uterine fluid of unilaterally-pregnant ewes[a]

| Day of pregnancy | Uterine fluid (ml) | DPPIV activity (mU/ml) | Total activity (mU) |
|---|---|---|---|
| 60 | 71 $\pm$ 23.2 | 3.2 $\pm$ 1.0 | 185 $\pm$ 38 |
| 100 | 105 $\pm$ 17.1 | 17.7 $\pm$ 4.1 | 1749 $\pm$ 370 |
| 140 | 337 $\pm$ 168.9 | 20.4 $\pm$ 5.0 | 8060 $\pm$5677 |

Values are means $\pm$ SEM (n = 3). Data were log-transformed before analysis.
[a]Day of pregnancy affected uterine fluid volume ($P = 0.08$), enzyme concentration ($P < 0.01$) and total enzyme activity ($P < 0.005$)

Table 5-2. Effect of progesterone on activity of dipeptidyl peptidase IV in uterine flushes of ewes and cows

| Treatment | Total dipeptidyl peptidase IV (mU)[a] |
|---|---|
| Ewes[a] | |
| CO | 2.09 ± 1.1 |
| P4-30 | 22.9 ± 3.5 |
| P4-60 | 804.9 ± 622.4 |
| Cows[b] | |
| CO | 7.2 ± 9.4 |
| P4-12 | 8.5 ± 7.6 |
| P4-30 | 135.7 ± 7.6 |

[a]Values are means ± SEM. Data were log-transformed before analysis. Ewes were treated with vehicle (CO) or progesterone for 30 (P4- 30) or 60 days (P4-60). Control vs progesterone, $P < 0.01$; P4-30 vs P4-60, $P < 0.01$; overall treatment effect, $P < 0.005$.

b Values are least-squares means ± SEM. Cows were treated with vehicle (CO) or progesterone for 12 (P4-12) or 30 days (P4-30). Control vs progesterone, $P < 0.005$; P4-12 vs P4-30, $P < 0.001$; overall treatment effect, $P < 0.001$.

proteins in the uterine fluid, UTM-proteins, which bind tightly to CM-Sepharose. Non-bound fractions were placed over a column of concanavalin A Sepharose (Figure 5-1). Enzymatic activity was completely retained by the column and was eluted with buffer containing methyl-$\alpha$-D-glucoside. Upon further separation by gel filtration using Sephacryl S-200 HR, DPPIV eluted as a single peak of $M_r$ = 140,000 kDa. Fractions rich in DPPIV were pooled and then further purified using a Gly-pro-affinity column (Figure 5-2). DPPIV bound to the column and was eluted by a 0.05 to 0.2 M NaCl gradient in 10 mM Tris-HCl (pH 8.2).

The yield and purification of DPPIV achieved is summarized in Table 5-3. DPPIV was purified approximately 3200 fold by the procedures utilized and 42.4% of the original activity was recovered. Analysis of the final DPPIV preparation by SDS-PAGE indicated the presence of several bands including one band at 107 kDa that was tentatively identified as DPPIV (Figure 5-2).

Table 5-3. Purification of DPPIV from sheep uterine fluid

| | Volume (ml) | Activity (mU/ml) | Total (mU) | Protein (mg/ml) | Total protein (mg) | Specific activity (mU/mg) | Purification | Yield (%) |
|---|---|---|---|---|---|---|---|---|
| Uterine fluid | 10 | 28.5 | 285 | 11.15 | 111.5 | 2.55 | 1 | 100 |
| CMS | 64.4 | 3.3 | 211.2 | .278 | 17.9 | 11.8 | 4.68 | 75 |
| Con A column | 2.5 | 67.4 | 168.4 | .175 | .438 | 382 | 153 | 60 |
| Sephacryl S-200 HR | 8.1 | 16.9 | 137.4 | .0067 | .054 | 2747.4 | 1090 | 49 |
| Gly-pro affinity | 4.2 | 28.6 | 121. | .0035 | .0147 | 8074.7 | 3203 | 42 |

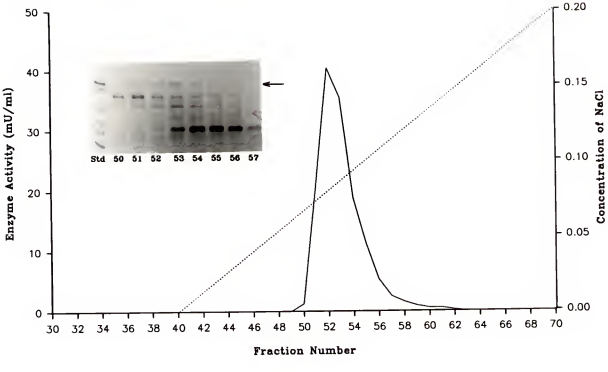


Figure 5-2. Purification of DPPIV using affinity chromatography on Gly-pro-NH-$(CH_2)_6$-NH-Sepharose 4B. The DPPIV bound to the column and was eluted with a linear gradient of NaCl (0-0.2 M) (……). Enzyme was measured using Gly-pro-*p*-nitroanilide as substrate (_____). The insert represents a Coomassie stained electrophoretogram of fractions 50-57 as resolved by SDS-PAGE. Molecular weight standards were $M_r$ = 106, 80, 49.5, 32.5, 27.5 and 18.5 kDa (from top to bottom). Note that active fractions contained a band of protein at 107 kDa (arrow) tentatively identified as DPPIV that was proportional in intensity to enzymatic activity of the fractions.

Enzymatic Properties of DPPIV

Using Gly-pro-*p*-nitroanilide as substrate, the optimum pH for DPPIV enzymatic activity was pH 8.3 when 0.05 M citric acid-$Na_2HCO_4$-borate was used as buffer at room temperature. The $K_m$ for Gly-pro-*p*-nitroanilide in Tris-HCl at pH 8.2 was estimated as 0.32 mM. As shown in Figure 5-3, enzymatic activity of DPPIV was not inhibited by UTM-proteins, a member of the serpin superfamily of serine proteinase inhibitors found in uterine fluid.

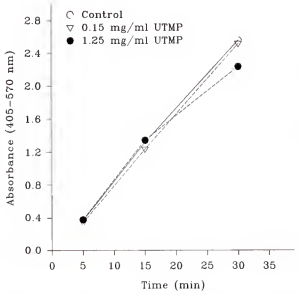


Figure 5-3. Effect of uterine milk proteins (UTM-proteins) on DPPIV enzymatic activity. The assay was carried out by incubating DPPIV and Gly-pro-*p*-nitroanilide with purified UTM-proteins in 10 mM Tris-HCl containing 0.2 M NaCl, pH 8.2, at room temperature. Product was measured as absorbance at 405 nm - absorbance at 570 nm after 5, 15, 30 min of incubation.

Microsequencing

Two dimensional SDS-PAGE of enriched DPPIV revealed a protein of 107 kDa with a pI of 6.8 (Figure 5-4). This protein was transferred to an Immobilon membrane and N-terminal amino sequence analysis performed for 34 cycles.

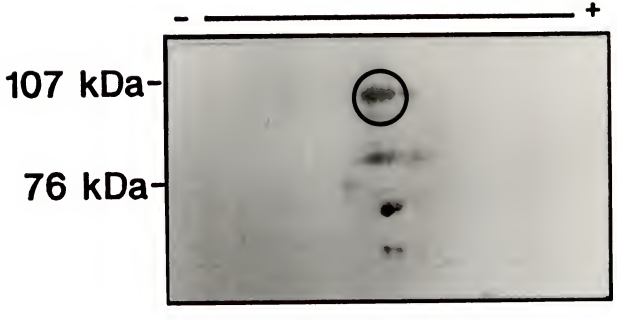


Figure 5-4 Resolution of an enriched preparation of DPPIV by 2-D SDS-PAGE. Shown is the pattern of proteins resolved by 2-D SDS-PAGE and transferred to Immobilon membrane from a preparation of DPPIV after purificationusing cation exchange, lectin, gel filtration and Gly-pro NH-$(CH_2)_6$-NH-Sepharose 4 B. Transferred proteins were visualized by staining with 0.1 % (w/v) Coomassie Blue R-250. Top spot (mol wt=107 kDa;pI 6.8) was excised and sequenced.

Search of the GenBank database revealed 77% sequence homology of this polypeptide to an internal region of human T-cell activation antigen CD26 beginning at position 39 (Figure 5-5). Similarly, there was 77% and 87% homology, respectively between sheep uterine DPPIV and internal sequences (beginning at position 37) of two rat DPPIV molecules. The sheep protein also possessed 77% homology to an internal region of mouse CD26 and 83% homology to an internal sequence of pig DPPIV.

```
SHEEP DPPIV   1  S R R T Y T L A D Y L K N T F R M K F Y N L R W V S D H E Y L

HUMAN CD26   39  S R K T Y T L T D Y L K N T Y R L K L Y S L R W I S D H E Y L

PIG DPPIV    37  S R R T Y T L T D Y L K S T F R V K F Y T L Q X I S D H E Y L

RAT DPPIV    37  S R R T Y T L A D Y L K N T F R V K S Y S L R W V S D S E Y L

MOUSE CD26   37  S R R T Y T L A D Y L K S T F R V K S Y S L W W V S D F E Y L

RAT DPPIV    37  S R R T Y T L T D Y L X X T F R V K S Y T L
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Figure 5-5. Comparision of the N-terminal amino acid sequence of sheep uterine DPPIV with others DPPIVs. The number to the left of the sequence indicates the position of the first amino acid represented. The pig sequence (*) is unpublished and was obtained by GenBank (Accession number X73276; Thomsen PD, Qvist H, Markland L,Andersson L, Sjostrom H, Noren O 1994 Assignment of the dipeptidyl peptidase IV gene to pig chromosome 15q21).

Localization of DPPIV in Endometrium of Pregnant and Progesterone-Treated Ewes

In endometrium of pregnant sheep, monoclonal antibody to human CD26

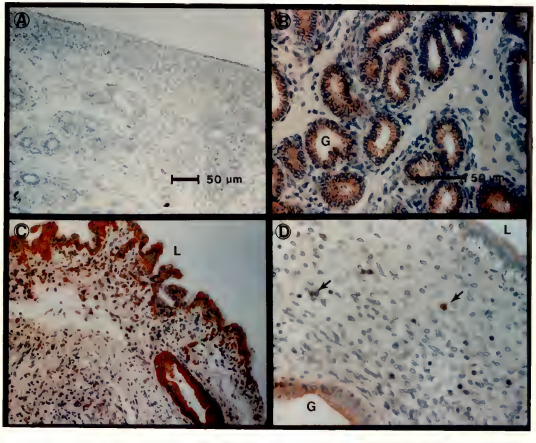


Figure 5-6. Immunohistochemical localization of DPPIV positive cells in the ovine endometrium using anti-CD26 monoclonal antibody. Sections were counterstained with hematoxylin. Panel A. Ovariectomized ewe treated with corn oil. Panel B. Ovariectomized ewe treated with progesterone for 60 d. Panel C. Pregnant ewe at d 140 of pregnancy. Panel D. Ewe at d 140 of pregnancy in which stromal cell staining was observed (arrows). G (gland), L (lumen).

reacted strongly with cells of the lumenal and glandular epithelium (Figure 5-6C).

For two pregnant ewes, some stromal cells were also present that reacted with the

antibody (Figure 5-6C and D). Reaction product for endometrium from progesterone-treated ewes was limited to epithelial cells only (Figure 5-6B). There was no positive staining in endometrial sections from ovariectomized ewes treated with corn oil (Figure 5-6A) or in sections in which control ascites fluid was used in place of primary antibody (data not shown).

Effects of DPPIV on Lymphocyte Proliferation

An experiment was carried out to examine the effects of DPPIV on the proliferation of lymphocytes stimulated by PHA (Figure 5-7. left panel). Addition of DPPIV did not increase [$^3$H] thymidine incorporation by lymphocytes. Addition of PHA caused an increase in [$^3$H] thymidine incorporation and effects of PHA were enhanced when DPPIV was also present in medium ($P < 0.01$). Asecond experiment was undertaken to test whether UTM-proteins blocked proliferation of PHA+DPPIV treated lymphocytes. DPPIV enhanced proliferation of T lymphocytes stimulated by PHA ($P=0.10$), and UTM-proteins caused inhibition of PHA-stimulated lymphocytes ($P=0.08$). However, UTM-proteins did not inhibit the costimulatory effect of DPPIV on PHA-stimulated lymphocyte proliferation (Figure 5-7. right panel).

## Discussion

The results of this study provide strong evidence that the progesterone-

dominated endometrium of the ewe and cow secrete an enzymatically-active form of

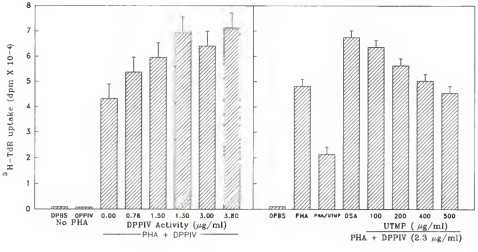


Figure 5-7. Effect of DPPIV and UTM-proteins on proliferation of sheep peripheral blood lymphocytes. The left panel represents the effect of DPPIV on PHA-stimulated lymphocyte proliferation. Lymphocytes were cultured without mitogen (DPBS) or with PHA + 0, 0.75, 1.5, 2.3, 3.0, 3.8 $\mu$/ml of DPPIV. DPPIV caused a linear dose-dependent ($P<0.01$) stimulation of proliferation. The right panel represents effects of UTM-proteins on enhancement of PHA stimulated-lymphocyte proliferation by uterine DPPIV. UTM-proteins (500 $\mu$g/ml) inhibited proliferation in the absence of DPPIV (compare PHA to PHA/UTM-proteins) and in the presence of DPPIV (compare OSA to 100, 200, 400 and 500 $\mu$g/ml UTM-proteins; linear effect of concentration, $P < 0.01$). Similarly, DPPIV increased proliferation in the absence of UTM-proteins (compare PHA to PHA+ DPPIV + OSA) and in the presence of UTM-proteins (compare PHA/UTM-proteins to PHA + DPPIV + 500 $\mu$g/ml UTM-proteins). When only four groups were analyzed (PHA, PHA/UTM-proteins (500 $\mu$g/ml), PHA + DPPIV (2.3 $\mu$g/ml) + OSA (500 $\mu$g/ml), and PHA + DPPIV + UTM-proteins), the DPPIV x UTM-proteins interaction was not significant, suggesting that the two molecules affected proliferation independently.

DPPIV that is probably derived from epithelial cells and, to a lesser extent, stromal cells. Thus, DPPIV should be considered among the progesterone-induced secretory

proteins of the uterus. Moreover, uterine DPPIV is capable of regulating lymphocyte function and may therefore play an important role in uterine biology during pregnancy. There are several lines of evidence to indicate that the DPPIV described in the uterus of the pregnant sheep is similar structurally and functionally to DPPIV molecules described elsewhere. First, the estimate of molecular weight, pH optimum and $K_m$ towards Gly-pro-*p*-nitroaniline is similar to published reports for other DPPIV molecules (MacNair and Kenny,1979; Kojma et al., 1989; Meester et al., 1992; Tanaka et al., 1994). Like CD26, which also displays DPPIV enzymatic activity (Tanaka et al., 1993), uterine DPPIV can co-activate proliferation of mitogen-stimulated lymphocytes. Additionally, monoclonal antibody to human CD26 reacts with epithelial and stromal cells of the sheep endometrium. Finally, the N-terminal amino acid sequence of the 107 kDa DPPIV isolated from uterine fluid of unilaterally-pregnant ewes possessed a high degree of homology with other DPPIV sequences. Comparison of the N-terminal amino acid sequence of the sheep uterine DPPIV identified by SDS-PAGE with sequences from other species revealed that the leader sequence was missing for the uterine DPPIV. Specifically, the sequence lacks the first 37-39 amino acids. These amino acids have been identified in human CD26 antigen as a short six-amino acid cytoplasmic domain and a region of hydrophobic amino acids between residue 7 and 28 that forms the cellmembrane-anchoring domain (Tanaka et al., 1992; Fleischer, 1994). It is likely

that DPPIV in uterine fluid is secreted either because the leader sequence is cleaved

after the protein is inserted into the plasma membrane, releasing its truncated form into uterine lumen, or because the leader sequence is cleaved after translocation of the protein into the lumen of endoplasmic reticulum. Other truncated forms of DPPIV exist in rat liver (MacCaughhan et al., 1990). It is not known whether membrane-bound forms of DPPIV are present in the endometrium although some or all of the DPPIV that reacted with anti human CD26 in histological sections could represent membrane DPPIV.

Previously, DPPIV has been identified in the uterus of rabbits and mice (Classen et al., 1987; Ohta et al., 1992). Secretion of DPPIV in the uterus of ewes and cows is under the regulation of progesterone because amounts of enzyme were increased in ovariectomized animals after treatment with progesterone and increased as pregnancy progressed. Only a few progesterone-induced proteins have been described in the uterus of the sheep and cow. Other examples include UTM-proteins (Moffatt et al., 1987; Leslie and Hansen, 1991), $\beta$-hexosaminidase (Hanesn et al., 1985), retinol binding protein (Thomas et al., 1992) and $\beta_2$-microglobulin (Vallet et al., 1991). Many endometrial secretory proteins are synthesized by epithelial cells. These cells are also the major site of uterine DPPIV but DPPIV-positive stromal cells were also sometimes found in the pregnant endometrium. These latter cells are likely to be lymphocytes. It is not known whether these stromal cells secrete DPPIV or whether the protein is a membrane-bound form.

The physiological functions of DPPIV in the uterus during pregnancy are

unknown. Collagen and fibronectin (Fleischer, 1994) are potential DPPIV substrates and DPPIV has been implicated in the initial phase of fibronectin-mediated cell spreading on collagen (Hanski et al., 1985). Thus, endometrial DPPIV could contribute to the remodelling of endometrial or placental structures during placentation. Other potential substrates for DPPIV include human chorionic gonadotropin and interleukin-1 (IL-1) (Fleischer, 1994). Endometrial DPPIV may also be involved in lymphocyte activation or migration. The endometrium of the sheep contains several subpopulations of intraepithelial lymphocytes and certain populations, in particular a $\gamma/\delta$ T-cell receptor$^+$ CD8$^+$ CD45R$^+$ population, shows a large increase in number as gestation advances (Lee et al., 1992; Meeusen et al., 1993). This increase could be caused by DPPIV because endometrial DPPIV costimulated PHA-stimulated peripheral blood lymphocyte proliferation. This effect of endometrial DPPIV is consistent with its homology to CD26, a 105 kDa human T cell surface molecule, that is strongly expressed on IL 2-dependent T cell lines and clones and a substantial proportion of activated T cells (Hegen et al., 1990; Kameoka et al., 1993; Fleischer, 1994;). Even though CD26 is a membrane bound protein, its cytoplasmic domain of six amino acids is too small to play a role in signal transduction (Tanaka et al., 1992) and soluble forms of CD26 can bind the signal transduction molecule, adenosine deaminase (ADA), (Kameoka et al., 1993) and cause T cell coactivation (Tanaka et al., 1994). Costimulatory activity of CD26 involves the association with other membrane proteins. For example, CD3 is a key element

in T cell activation and the costimulatory activity of CD26 (Dang et al., 1990). CD26 also associates with CD45 (Torimoto et al., 1991) and with ADA (Kameoka et al., 1993). DPPIV also induces synthesis of cytokines such as IL-1$\beta$, IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin (Feltke et al., 1991). Some of these, notably IL-1 and GM-CSF, have been implicated in embryonic and uterine function (Betts and Hansen, 1992; Imakawa et al., 1992) and it is possible that uterine DPPIV enhances secretion of these molecules from endometrial lymphocytes and other cells. DPPIV also could act as an adhesion molecule to cause interactions between T lymphocytes and extracellular matrix that lead to T-cell activation (Dang et al., 1990).

In contrast to DPPIV, which stimulates lymphocyte proliferation, the predominant proteins in uterine secretions during pregnancy are the UTM-proteins, which inhibit a variety of cellular immune responses including PHA-induced sheep lymphocyte proliferation (Skopets and Hansen, 1993) and sheep and mouse NK cell activity (Liu and Hansen, 1993). It is likely the actions of UTM-proteins predominate in the uterus because crude uterine fluid, which contains both UTM-proteins and DPPIV, strongly inhibits lymphocyte proliferation (Stephenson and Hansen, 1990). DPPIV increased proliferation of UTM-proteins-treated lymphocytes in the present study, but such lymphocytes were less active than lymphocytes not treated with UTM-proteins. It is possible, however, that actions of DPPIV could predominate in certain subpopulations of lymphocytes that might be resistant to actions of UTM-

proteins. Also, stromal lymphocytes may not be exposed to UTM-proteins, which are of epithelial cell origin (Leslie and Hansen, 1991).

Coactivation of lymphocytes by DPPIV can be blocked by inhibitors of DPPIV enzymatic activity (Tanaka et al., 1993; Tanaka et al., 1994), indicating that the enzymatic activity of CD26 is required for coactivation. This concept has been questioned, however (Fleischer, 1994). Since DPPIV is a serine protease (Walter et al., 1980) and the UTM-proteins are members of the serpin superfamily of serine protease inhibitors (Ing and Roberts, 1989), it was hypothesized that UTM-proteins might block coactivation of lymphocytes by endometrial DPPIV through inhibition of DPPIV enzymatic activity. However, UTM-proteins did not inhibit the catalytic activity of endometrial DPPIV nor prevent induction of coactivation. To date, no protease has been found that is susceptible to UTM-proteins inhibition (Ing and Roberts, 1989) and UTM-proteins may be an inactive serpin. Furthermore, present data indicate that, while UTM-proteins antagonizes the overall effect of DPPIV (inhibits lymphocyte proliferation rather than stimulates proliferation), its actions are independent of those of DPPIV. Thus, UTM-proteins does not interfere with crosslinking of DPPIV to CD3, CD45 or ADA (Hegen et al., 1990; Dang et al., 1990) or block the resultant signal transduction.

In conclusion, present results indicate that ovine and bovine endometrium secrete DPPIV in the uterine lumen in response to progesterone. The protein was identified in the sheep as a 107 kDa uterine protein that was homologous to the human

T-lymphocyte activation antigen. DPPIV is synthesized by lumenal and glandular endometrial epithelial cells of pregnant and progesterone-treated sheep and in some stromal cells of pregnant sheep endometrium. This soluble form of uterine DPPIV costimulates proliferation of mitogen-stimulated sheep peripheral blood lymphocytes and may therefore be involved in the regulation of immune function in the uterus. Alternatively, the protease serves an as yet unknown role as an intrauterine protease.

# CHAPTER VI
# MODULATION OF EXPRESSION OF CELL SURFACE MARKERS ON $\gamma\delta$ TCR$^+$ INRAEPITHELIAL LYMPHOCYTES IN THE OVINE ENDOMETRIUM BY LOCAL AND SYSTEMIC EFFECTS OF PREGNANCY

## Introduction

Lymphocytes bearing the $\gamma\delta$ T-cell receptor are a major lymphocyte population in many epithelia including the endometrial epithelium of the uterus (Yeh et al., 1990; Heyborne et al., 1992; Mincheva-Nilsson et al., 1992; Meeusen et al., 1993). The function of $\gamma\delta$ TCR$^+$ intraepithelial lymphocytes (IEL) in the uterine endometrium is not clear. One possibility is that, like other $\gamma\delta$ TCR$^+$ lymphocytes, they play a role in recognition of microbial antigens and tumor cells (Meeusen et al., 1993). Endometrial $\gamma\delta$ TCR$^+$ lymphocytes could also react with trophoblast through actions that either compromise or enhance placental function. Unlike T-lymphocytes of $\alpha\beta$-TCR lineage, $\gamma\delta$ TCR$^+$ lymphocytes do not readily recognize MHC proteins and are not usually involved in allograft responses (Hass et al., 1993; Eto et al., 1994). Furthermore, expression of classical MHC proteins on placenta is limited or absent (Templeton et al., 1987; Low et al., 1990). Thus, it is unlikely that endometrial $\gamma\delta$ TCR$^+$ lymphocytes participate in rejection responses directed towards conceptus MHC antigens. Nonetheless, $\gamma\delta$ TCR$^+$ lymphocytes could pose a threat towards the conceptus since activated $\gamma\delta$ TCR$^+$ cells can exhibit properties characteristic of non-

MHC restricted LAK cells (Maziarz et al., 1991) and LAK cells can lyse trophoblast tissue (King and Loke, 1993; Segerson and Gunsett, 1994). Conversely, there is evidence that endometrial $\gamma\delta$ $TCR^+$ IEL can suppress reactivity of endometrial IEL to fetal alloantigens in mice (Suzuki et al., 1995). Also, mouse $\gamma\delta$ $TCR^+$ lymphocytes released IL-2/IL-4 bioactivity in response to trophoblast in a manner independent of trophoblast MHC antigen expression (Heyborne et al., 1994) and it is possible that these cytokines or other growth factors and cytokines released by $\gamma\delta$ $TCR^+$ lymphocytes (Morita et al., 1991; Boismenu et al., 1994) contribute to trophoblast growth and differentiation.

Pregnancy in mice results in an increase in the number and activation status of $\gamma\delta$ $TCR^+$ lymphocytes within the uterus (Heyborne et al., 1992; Suzuki et al., 1995; Kimura et al., 1995). In the sheep, the type of lymphocytes resident within the endometrium depends upon the placental region that the endometrium is in contact with. Intimate attachment of placenta and endometrium occurs at specialized knob-like structures called placentomes - after day 30 of gestation, there are few lymphocytes in the placentomal regions of the endometrium (Gogolin-Ewens et al., 1989). As pregnancy proceeds, there is also a decrease in numbers of IEL in the glandular epithelium of the interplacentomal region of the endometrium (Gogolin-Ewens et al., 1989; Lee et al., 1992). In contrast, the number of lymphocytes in the luminal epithelium of the interplacentomal endometrium increases dramatically after day 50 of pregnancy. This increase in number of lymphocytes is caused primarily by

increased numbers of granulated lymphocytes possessing a $CD45R^+CD8^+$ $\gamma\delta$ $TCR^+$ phenotype (Meeusen et al., 1993; Lee et al., 1992). The increase in number of granulated lymphocytes is accompanied by a concomitant increase in the size and number of granules (Meeusen et al., 1993; Gogolin-Ewens et al., 1989; Lee et al., 1992).

It is unknown whether the pregnancy-associated activation of endometrial $\gamma\delta$ $TCR^+$ IEL occurs as a result of systemic changes in pregnancy hormones or as result of local activation by the placenta. Local stimulation by trophoblast may involve allogeneic antigens because the relative number of $\gamma\delta$ $TCR^+$ lymphocytes recovered from uteri of pregnant mice was greater for allogeneic pregnancies than for syngeneic pregnancies (Suzuki et al., 1995). MHC-independent recognition of trophoblast by endometrial $\gamma\delta$ $TcR^+$ IEL has also been reported (Heyborne et al., 1994). Changes in numbers and function of endometrial IEL are likely under the control of locally-produced soluble regulators of lymphocyte function induced by systemic hormones of pregnancy. Foremost among these in the sheep are the uterine milk proteins (UTM-proteins), a pair of progesterone-induced glycoproteins of the serpin superfamily of serine protease inhibitors that are produced by endometrial epithelial cells during pregnancy (Moffatt et al., 1987) and which inhibit a variety of lymphocyte functions in vitro and in vivo (Skopets and Hansen, 1993; Liu and Hansen, 1993; Skopets et al., 1995).

The unilaterally-pregnant ewe is an ideal model to separate systemic effects of pregnancy from local effects of the conceptus. In this model, pregnancy is limited to the non-ligated uterine horn, allowing the pregnant uterine horn exposure to hormones of pregnancy and the local effects of the conceptus. In contrast, the uterine horn not containing the conceptus is only exposed to systemic changes accompanying pregnancy. Gestation proceeds normally (Bazer et al., 1979) and the ligated uterine horn has the same protein secretory pattern as the pregnant uterine horn (Moffatt et al., 1987; Stephenson et al., 1989). The goal of the present study was to use this model to determine the relative contribution of local versus systemic regulation of endometrial $\gamma\delta$ $TCR^+$ IEL during pregnancy.

## Materials and Methods

### Monoclonal Antibodies

Hybridomas producing antibodies to $\gamma\delta$ TCR (86D), CD44 (25-32), CD29 (47) and L-selectin (Du129) were purchased from the European Collection of Animal Cell Culture, Salisbury, UK. The anti-CD25 monoclonal antibody was kindly provided as ascites fluid by Dr. Andrew D. Nash, Centre for Animal Biotechnology, School of Veterinary Science, The University of Melbourne, Parkville, Australia. For other antibodies, ascites were prepared from Balb/c mice.

### Purification of Monoclonal Antibodies

Except for antibody to CD25, which was used as ascites fluid, antibodies were purified before use. Ascites fluid was diluted 1:10 (v/v) with 1.5 M glycine-NaOH

(pH 8.9) containing 3 M NaCl and loaded onto a 10 ml column of Protein A-agarose (Bio-Rad, Richmond, CA) equilibrated with the same buffer. Column chromatography was performed using FPLC (Pharmacia, Piscataway, NJ). Immunoglobulin was eluted from the column using a linear pH gradient (pH 5.0 - 7.8) of 0.1 M citric acid/0.1 M phosphate buffer and a flow rate of 0.5 ml/min. Purity was determined by 10% SDS-PAGE under reduced conditions using Coomassie blue as a protein stain.

Biotinylation of the Anti γδ TCR mAb

Purified IgG (2 mg/ml in 0.1 M sodium acetate buffer, pH 5.5) was mixed with fresh 20 mM sodium meta-periodate for 20 min on ice. After oxidation, IgG was dialyzed against 0.1 M sodium acetate buffer (pH 5.5) overnight. Biotin hydrazide (Pierce, Rockford, IL) was added to the IgG solution at a final concentration of 1.2 mg/ml. After 2 h at room temperature, biotinylated IgG was dialyzed against the sodium acetate buffer overnight. The antibody working concentration was determined using flow cytometry.

Animals

Four adult, multiparous Rambouillet ewes were made unilaterally-pregnant as described (Bazer et al., 1979). Briefly, one uterine horn was ligated and the ipsilateral ovary was removed surgically. Ewes were bred at estrus via natural mating to fertile rams. The resultant pregnancy was limited to the non-ligated uterine horn, hereafter referred to as the pregnant uterus-pregnant horn (PU-PH). The ligated

uterine horn was exposed to hormones of pregnancy but did not contain fetal placental tissue and is hereafter referred to as the pregnant uterus-nonpregnant horn (PU-NPH). Additionally, four cyclic ewes of similar breeding and parity were used as controls. Ewes were sacrificed at an abattoir at either day 140 of gestation (pregnant ewes) or at randomly chosen stages of the estrous cycle (cyclic ewes). All cyclic ewes had a corpus luteum on one ovary at slaughter, indicating that ewes were in the luteal phase of the estrous cycle. Uteri were removed and taken to the laboratory for processing.

Isolation of IEL

The uterine endometrium was exposed by bisecting the uterine wall. Placental tissues were removed and the endometrium was collected by dissection and washed in Dulbecco's phosphate-buffered saline, pH 7.4 (DPBS). Epithelial cells were removed by gentle scraping with a scalpel blade and suspended in DPBS containing 5 mM EDTA. The epithelial cell suspension was stirred for 2 h at room temperature to facilitate disaggregation of epithelial clumps. The cell suspension was washed twice in DPBS and then filtered through a 40 $\mu$m nylon mesh to remove remaining epithelial clumps. Cells passing through the filter were counted with a hemocytometer and adjusted to a concentration of 1 x $10^7$ cells/ml in staining buffer (DPBS containing 1% BSA (w/v) and 1 mM EDTA).

Isolation of PBL

Jugular blood was collected immediately prior to slaughter. PBL were obtained following purification using Ficoll-Hypaque (Sigma, St. Louis, MO) as

described (21). Cells were resuspended to a concentration of 1 x $10^7$/ ml in staining buffer.

Immunofluorescence Analysis

Antibodies were diluted in staining buffer and were used according to the optimal concentration determined by titration analysis (20-40 $\mu$g/ml). Cells (1 x $10^6$ ) were incubated with 100 $\mu$l primary antibody for 30 min on ice, washed with DPBS, and incubated for another 30 min on ice with 100 $\mu$l second antibody coupled to FITC (anti-mouse IgG whole molecule, Sigma, St. Louis, MO). Cells were washed and incubated with 100 $\mu$l biotinylated antibody against $\gamma\delta$ TCR for 30 min on ice, and then reacted with 5 $\mu$g streptavidin-phycoerythrin (Sigma, St Louis, MO) in 100 $\mu$l staining buffer for 10 min on ice. Immunofluorescent analysis was performed using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA).

Statistical Analysis

Data were collected using the PC-LYSIS program, version 2.0 (Becton Dickinson, San Jose, CA). The effect of treatments on proportion of $\gamma\delta$ $TCR^+$ lymphocytes staining positive for CD25, CD44, CD29, and L-selectin, fluorescent intensity of positive staining cells, and degree of granularity of $\gamma\delta$ $TCR^+$ IEL and PBL were assessed by least-squares analysis of variance using the GLM procedure of the Statistical Analysis System (SAS, 1989). Main effects included physiological status, ewe nested within status, and tissue. Ewe was considered as a random effect

and other main effects were considered as fixed. Data were analyzed several ways because design limitations (three tissues were analyzed for pregnant animals and two tissues for cyclic animals) limited determination of all treatment effects in one analysis. Accordingly, data were analyzed as a complete data set, and as various subsets of the data (i.e., PBL only, cyclic animals only, uterine tissues only, etc.). Orthogonal contrasts were used to separate main effects of tissue into single degree of freedom comparisons (PBL vs IEL; PU-PH vs PU-NPH).

## Results

### Patterns of Immunofluorescent Staining

Analysis of immunofluorescent staining patterns was conducted after gatingcellsaccording to forward and side scatter. The gate for IEL was set wide so that most $\gamma\delta$ $TCR^+$ cells were included. Using windows based on fluorescence of cells stained with isotype controls, gated lymphocytes from peripheral blood (Figure 6-1)

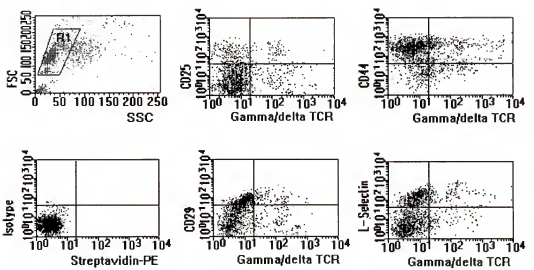


Figure 6-1. Representative FACS analysis of PBL from pregnant ewe. The upper left panels shows distribution of cells according to forward scatter and side scatter. The area labeled R1 represents the gated area used for further analysis. The lower left panel represents the staining patterns where isotype control and streptavidin-PE were used instead of antibodies. Other panels represent two-color fluorescence patterns where cells were labeled with 1) primary antibody (anti-CD25, CD44, CD29 or L-selectin) followed by anti-mouse IgG coupled to FITC and 2) biotinylated antibody to $\gamma\delta$ TCR followed by streptavidin-phycoerythrin.

and uterine luminal epithelium (Figure 6-2) could be separated into $\gamma\delta$ $TCR^+$ and $\gamma\delta$ $TCR^-$ populations. Cells were also separated into those positive and negative for CD25, CD44, CD29, and L-selectin. Positive and negative populations could be

discerned except that few L-selectin$^+$ cells were present in preparations of endometrial IEL (Figure 6-2).

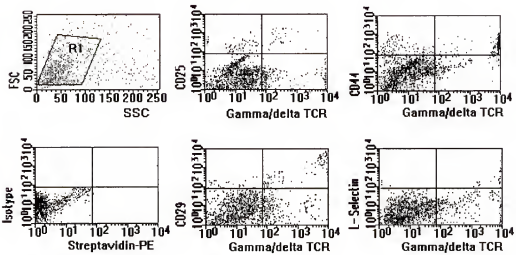


Figure 6-2. Representative FACS analysis of IEL from PU-PH. The upper left panels shows distribution of cells according to forward scatter and side scatter. The area labeled R1 represents the gated area used for further analysis. The lower left panel represents the staining patterns where isotype controls for FITC and streptavidin-PE were used instead of antibodies. Other panels represent two-color fluorescence patterns where cells were labeled with 1) primary antibody (anti-CD25, CD44, CD29 or L-selectin) followed by anti-mouse IgG coupled to FITC and 2) biotinylated antibody to $\gamma\delta$ TCR followed by streptavidin-phycoerythrin.

Proportions of $\gamma\delta$ TCR$^+$ Cells Staining Positive for CD25, CD44, CD29 and L-selectin

The proportion of γδ TCR$^+$ cells staining positive for CD25 was generally lower for IEL than for PBL (P=0.11 if exclude PU-PH) except for IEL recovered from the PU-PH (Figure 6-3). In fact, more γδ TCR$^+$ IEL from the PU-PH were positive for CD25 than for IEL from other tissues or for PBL. The difference in

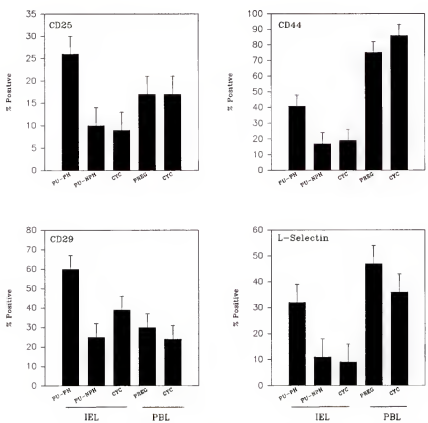


Figure 6-3. Relative proportion of γδ TCR$^+$ cells staining positive for CD25, CD44, CD29 and L-selectin. Abbreviations are as follows: PU-PH, pregnant uterus-pregnant uterine horn; PU-NPH, pregnant uterus-nonpregnant uterine horn; CYC, cyclic; PREG, pregnant. Results represent the least-squares means ± SEM of data from four ewes per group.

proportion of $CD25^+$ cells between PU-PH and PU-NPH was significant ($P<0.05$), indicating activation of CD25 expression induced by local presence of the conceptus.

The percentage of $\gamma\delta$ $TCR^+$cells positive for CD44 was lower ($P<0.001$) for IEL than for PBL of all tissues (Figure 6-3). While there was no difference in the percentage of $CD44^+$ IEL between $\gamma\delta$ $TCR^+$ cells of cyclic ewes as compared to $\gamma\delta$ $TCR^+$ cells from the PU-NPH, more IEL from the PU-PH expressed CD44 than from the PU-NPH ($P=0.08$). The proportion of $\gamma\delta$ $TCR^+$ lymphocytes staining positive for CD29 was generally similar between tissues except that a greater proportion of $\gamma\delta$ $TCR^+$ cells from PU-PH were positive for CD29 than for other tissues (PU-PH vs PU-NPH; $P=0.10$).

Fewer $\gamma\delta$ $TCR^+$ from IEL were positive for L-selectin as compared to $\gamma\delta$ $TCR^+$ from PBL ($P<0.01$) (Figure 6-3). The percentage of cells positive for L-selectin was similar for IEL from cyclic uteri and PU-NPH; local presence of the conceptus stimulated L-selectin expression as indicated by the fact that a greater ($P=0.08$) proportion of $\gamma\delta$ $TCR^+$ cells from IEL of PU-PH were positive for L-selectin than for $\gamma\delta$ $TCR^+$ cells from IEL of PU-NPH.

Intensity of Fluorescence

Fluorescent intensity associated with CD25, which is a measure of the number of molecules on the cell surface accessible to antibody, was greater ($P=0.10$) for $\gamma\delta$ $TCR^+$ IEL than for $\gamma\delta$ $TCR^+$ PBL (Figure 6-4). While intensity

tended to be greater for IEL from the PU-PH than IEL from the PU-NPH, this difference was not significant. Pregnancy did not affect the expression of CD25 on $\gamma\delta$ TCR$^+$ PBL (Figure 6-4). Fluorescent intensity of CD44, CD29 and L-selectin for $\gamma\delta$ TCR$^+$ cells was generally similar between groups (results not shown). The one striking difference was for L-selectin fluorescent intensity - positive cells from IEL of cyclic ewes (least squares mean =433), which were rare, had higher intensity than for positive cells from other tissues (least squares means = 189 for IEL from PU-NPH, 165 for IEL from PU-PH, 144 for PBL from pregnant ewes and 112 for PBL

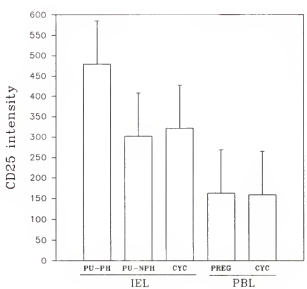


Figure 6-4. Intensity of immunofluorescence associated with CD25 for $\gamma\delta$ TcR$^+$CD25$^+$ IEL and PBL. Abbreviations are as follows: PU-PH, pregnant uterus-pregnant uterine horn; PU-NPH, pregnant uterus-nonpregnant uterine horn; CYC, cyclic; PREG, pregnant. IEL= intraepithelial lymphocytes; PBL=peripheral blood lymphocytes. Results represent the least-squares means $\pm$ SEM of data from four ewes per group.

from cyclic ewes, SEM=25; tissue, $P<0.001$; pregnancy x tissue, $P<0.01$).

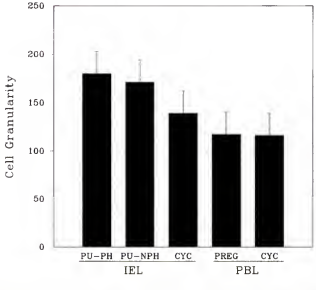


Figure 6-5. Granularity of γδ TCR$^+$ IEL and PBL as determined by side scatter. Abbreviations are as follows: PU-PH, pregnant uterus-pregnant uterine horn; PU-NPH, pregnant uterus-nonpregnant uterine horn; CYC, cyclic; PREG, pregnant. Results represent the least-squares means ± SEM of data from four ewes per group.

Granularity of γδ TCR$^+$ Lymphocytes

Side scatter of light was used to estimate granularity of γδ TCR$^+$ cells (Figure 6-5). There was a tendency for granularity of γδ TCR$^+$ IEL to be higher during pregnancy but this difference was not significant. Nonetheless, granularity of IEL from both uterine horns of pregnant ewes was higher than granularity of γδ TCR$^+$

PBL from pregnant ewes ($P=0.07$). In contrast, granularity of $\gamma\delta$ $TCR^+$ cells from cyclic ewes was similar for IEL and PBL.

## Discussion

These data provide evidence that pregnancy is associated with changes in function of $\gamma\delta$ $TCR^+$ IEL from the luminal epithelium and this effect of pregnancy is due primarily to the local presence of the conceptus rather than to systemic hormonal changes associated with pregnancy. In particular, a larger proportion of endometrial $\gamma\delta$ $TCR^+$ IEL recovered from the uterine horn containing the conceptus were positive for CD25, CD44, CD29 and L-selectin than endometrial $\gamma\delta$ $TCR^+$ IEL recovered from the nonpregnant uterine horn of pregnant ewes or from the uterus of cyclic ewes. Increased expression of these cell surface markers is consistent with increased activation (Verhagen et al., 1993; Ramsdell et al., 1994) or maturation (Tatsumi et al., 1993). The molecule from trophoblast causing activation of endometrial $\gamma\delta$ $TCR^+$ IEL is not clear. Classical MHC antigens are unlikely to represent the activation/maturation signal because $\gamma\delta$ $TCR^+$ lymphocytes do not readily recognize MHC antigens (Haas et al., 1993), expression of classical MHC antigens on placenta is limited or absent (Templeton et al., 1987; Low et al., 1990) and activation of cultured $\gamma\delta$ $TCR^+$ mouse lymphocytes by trophoblast was not dependent upon MHC expression (Heyborne et al., 1994). One possibility is that endometrial $\gamma\delta$ $TCR^+$ IEL are activated through binding of vitronectin receptors on IEL to trophoblast extracellular matrix proteins - such binding can lead to antigen-independent activation

of $\gamma\delta$ TCR$^+$ T cells (Sturmhöfel et al., 1995). Also, $\gamma\delta$ TCR$^+$ lymphocytes can be activated by heat shock protein 60 (O'brien et al., 1991) and glandular epithelial cells of the pregnant human endometrium have been reported to express heat shock protein 60 on the basolateral side facing underlying $\gamma\delta$ TCR$^+$ cells (Mincheva-Nilsson et al., 1994).

The only evidence for systemic regulation of endometrial $\gamma\delta$ TCR$^+$ IEL is for granularity because side scatter of endometrial $\gamma\delta$ TCR$^+$ IEL tended to increase during pregnancy in both uterine horns. However, this difference was not significant and the large pregnancy-associated increase in granularity of endometrial $\gamma\delta$ TCR$^+$ lymphocytes reported earlier (Meeusen et al., 1993; Lee et al., 1992) may reflect changes in a more restricted population of $\gamma\delta$ TCR$^+$ IEL than measured in the present study.

The consequences of local or systemic stimulation of endometrial $\gamma\delta$ TCR$^+$ IEL for the conceptus are unclear because the role of these cells is unknown. Activation of $\gamma\delta$ TCR$^+$ IEL may be associated with transformation of these cells into LAK cells but there is no evidence for $\gamma\delta$ TCR$^+$ LAK cells in endometrial epithelium. One possibility for which evidence exists is that activated $\gamma\delta$ TCR$^+$ IEL could suppress other endometrial IEL from developing anti-conceptus cytotoxic responses (Suzuki et al., 1995). Also, $\gamma\delta$ TCR$^+$ IEL may produce cytokines that stimulate trophoblast growth or hormone secretion. Various cytokines have been described that can affect placental function including GM-CSF, IL-1 and CSF-1 (Imakawa et al.,

1993; Librach et al., 1994; Garcia-Lloret et al., 1994). Increased expression of cell adhesion molecules such as CD44, CD29 and L-selectin on the surface of endometrial $\gamma\delta$ TCR$^+$ IEL during pregnancy may enhance recognition of trophoblast by $\gamma\delta$ TCR$^+$ cells because the surface of the trophoblast is rich in molecules involved in cell adhesion (Cross et al., 1994).

The maternal-fetal interface of the pregnant ewe is bathed in several molecules which can inhibit lymphocyte function, including the UTM-proteins (Skopets and Hansen, 1993; Liu and Hansen, 1993; Skopets et al., 1995), prostaglandin $E_2$ (Malayer and Hansen, 1990), and proteins produced by the cotyledonary portion of the placenta (Low et al., 1991). Despite this plethora of putative lymphocyte-inhibitory substances, there was no evidence to indicate that $\gamma\delta$ TCR$^+$ IEL isolated from the nonpregnant uterine horn of unilaterally-pregnant ewes were inhibited as compared to similar cells isolated from the uterus of cyclic ewes. There are two possible reasons for this finding. One is that $\gamma\delta$ TCR$^+$ IEL from the nonpregnant uterine horn of unilaterally-pregnant ewes are quiescent and inhibitory substances would therefore not further reduce activity. Consistent with this possibility is the fact that few $\gamma\delta$ TCR$^+$ IEL from cyclic endometrium and the nonpregnant uterine horn of unilaterally-pregnant ewes were positive for CD25. Perhaps, the activation of $\gamma\delta$ TCR$^+$ IEL that occurred in the pregnant horn of unilaterally-pregnant ewes was limited by the presence of local immunosuppressants. A second possibility is that the major target cells for the immunoregulatory molecules listed above are $\alpha\beta$ TCR$^+$

lymphocytes and NK cells and that γδ TCR$^+$ IEL are resistant to inhibitory effects of these molecules. Such unresponsiveness may be important to the outcome of pregnancy if γδ TCR$^+$ IEL function to suppress anti-conceptus immune responses by other lymphocytes (Suzuki et al., 1995) or produce growth-promoting cytokines (32-34).

Except for cells isolated from the pregnant uterine horn, a lower proportion of γδ TCR$^+$ IEL expressed CD25, CD44 and L-selectin than γδ TCR$^+$ PBL. One plausible explanation for this finding is that most endometrial γδ TCR$^+$ IEL develop extrathymically rather than being derived from a circulating pool of γδ TCR$^+$ lymphocytes. Recent evidence suggests that uterine γδ TCR$^+$ cells in humans (Hayakawa et al., 1994) and mice (Kimura et al., 1995) express recombinase-activating genes-1 and-2. Also, certain observations are inconsistent with extravasation from blood being a major source of γδ TCR$^+$ IEL in sheep. Most importantly, there is a near absence of lymphocytes in the stromal compartment of the endometrium (Templeton et al., 1987; Gottshall and Hansen, 1992). Also, present results indicate expression of CD44, which has been implicated in lymphocyte homing to mucosal tissues (Salmi and Jalkanen, 1991), is low for endometrial γδ TCR$^+$ IEL. Finally, γδ TCR$^+$ IEL in sheep endometrium express CD45R (Meeusen et al., 1993), which is analogous to the CD45RA isotype characteristic of naive T cells (Mackay et al., 1990). If most endometrial γδ TCR$^+$ IEL arise by local maturation, it is likely that the increase in number and activation of uterine γδ TCR$^+$ lymphocytes seen in

pregnancy of mice (Heyborne et al., 1992; Susuki et al., 1995; Kimura et al., 1995) and sheep (4, present results) is a result of conceptus-driven maturation of $\gamma\delta$ $TCR^+$ precursors and immature cells.

The present studies were performed on IEL obtained from luminal epithelium. The glandular epithelium was not evaluated due to technical difficulties in obtaining these cells free from blood contamination. This region of endometrial epithelium also contains abundant IEL - available evidence from histological studies indicates that pregnancy-associated changes in IEL from glandular epithelium do not parallel those in luminal epithelium (Lee et al., 1992). While there is therefore a need to extend studies to IEL from glandular epithelium, the majority of large granular lymphocytes during late pregnancy that are characteristic of $\gamma\delta$ $TcR^+$ IEL are located in the luminal epithelium (Lee et al., 1992).

In conclusion, these results indicate that pregnancy is associated with increased expression of CD25, CD44, CD29 and L-selectin by endometrial $\gamma\delta$ $TCR^+$ IEL. The signal for upregulation of expression of these proteins is provided in a local manner by the conceptus. Additionally, pregnancy may cause an increase in granularity through a mechanism that does not require the local presence of the conceptus. The significance of these changes for function of $\gamma\delta$ $TCR^+$ IEL remains to be determined - in particular, it is not known whether the increase in cytokine secretion and trophoblast-lymphocyte adhesion that is likely to accompany changes in CD25, CD44 and L-selectin expression lead to positive effects on trophoblast growth and hormone

secretion or contribute to cytotoxic responses against that the trophoblast that must be limited by other regulatory processes.

# CHAPTER VII
# GENERAL DISCUSSION

There are four major conclusions related to the function of the UTM-proteins in immunoregulation during pregnancy that can be derived from work presented in this dissertation. First, studies in Chapter III indicate that UTM-proteins inhibit T cell proliferation induced by antigen, lectin and PMA. In addition, UTM-proteins down-regulate expression of the adhesion molecule CD44 that is involved in lymphocyte homing. These inhibitory effects presumably involve binding of UTM-proteins to the cell surface of lymphocytes. Specific binding of UTM-proteins to PBL was demonstrated and heparin, which blocks binding of UTM-proteins to PBL, also reduced lymphocyte-inhibitory effects of UTM-proteins. Other proteinase inhibitors such as $\alpha_1$-antitrypsin (Bata and Revillard, 1981) and $\alpha_2$-macroglobulin (Peterson et al., 1989) have lymphocyte-inhibitory effects through similar binding sites or receptors on the lymphocyte surface. The signal transduction system activated by UTM-proteins is unknown but results from Chapter III indicate that these inhibitory effects interfere with protein kinase C mediated T cell activation since UTM-proteins block PMA-induced lymphocyte proliferation. Similar actions have been described for human immunosuppressive pregnancy zone protein (Saito et al., 1990) and trophoblast-derived suppressor (Matsuzaki et al., 1991). Interestingly, UTM-proteins did not

block IL-2 mediated proliferation or prevent expression of IL-2 receptor coincident with T cell action. A hypothetical model for the molecular mechanisms by which

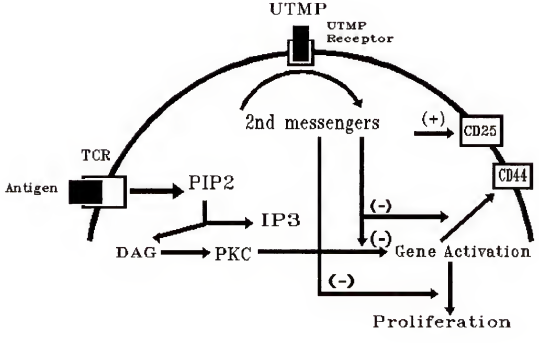


Figure 7-1 Model for inhibition of activated lymphocytes by UTMP (Uterine milk proteins). T cell receptor (TCR); Diacylglycerol (DAG); Inositol 1,4,5-trisphosphate ($IP_3$).

UTM-proteins regulate lymphocyte function is shown in Figure 7-1. Antigenic or lectin stimulation of T cells involves the formation of DAG and $IP_3$, leading to activation of PKC and increased intracellular concentration of free $Ca^{2+}$ (Barja et al.,

1994). The $Ca^{2+}$ signal pathway is responsible for IL-2-dependent T cell proliferation, while the PKC pathway induces transcription of T cell activation genes and is responsible for IL-2-independent T cell proliferation (Galron et al., 1994). This later response is mimicked by PMA. Based on present findings, UTM-proteins bind to the surface of lymphocytes to trigger production of second messengers which are responsible for blocking either PKC activation by DAG or by blocking some event down-stream from PKC activation. As a result, cell proliferation and CD44 expression are inhibited. For some reasons, UTM-proteins cause increased expression of CD25.

The second major conclusion of this dissertation is that UTM-proteins inhibit basal and poly(I).poly(C) activated NK cell activity and protect embryos from attack by activated NK cells (Chapter IV). This inhibitory effect on NK cells has been reported for other uterine immunosuppressive proteins like human decidual protein (Golander et al., 1981), human PP14 (Okamoto et al., 1991) and the serpin antithrombin III (Hudig et al., 1981). These results further implicate the UTM-proteins as important regulators of uterine endometrial lymphocyte function.

Like $\alpha_1$ antitrypsin, UTM-proteins block lymphocyte proliferation (Segerson et al., 1984; Zhang and Miller, 1989; Skopets and Hansen, 1993) and bind IgA (Hansen and Newton, 1988). While this suggests that the fact that UTM-proteins are serpins is relevant to the role as lymphocyte-inhibitory activity, it is not clear whether UTM-proteins exact this action by interacting with proteinases of region for "stressed" conformation that other serpins require for activity. Present results have not led to

identification of a function of UTM-proteins that is unique for serpins. UTM-proteins did not inhibit sheep uterine CD26 enzymatic activity or costimulatory actions on PHA-induced lymphocyte proliferation (Chapter IV). Interestingly, CD26 is a serine proteinase and a potential target for serpins and was found to be abundant in uterine secretions during pregnancy. Moreover, the UTM-proteins do not bind to PBL through the SECR that recognize serpin-enzyme complex and which has been implicated in regulation of cell functions (Potempa et al., 1994). Today, no target enzymes have been found to be inhibited by UTM-proteins (Ing and Roberts, 1989; Hansen and Liu, 1994) except for weak inhibition of the aspartic proteinase pepsin (Mathialagan et al., 1995). Many serpins with biological activity have no known antiproteinase activity; examples are maspin, which suppresses tumor growth (Zou et al., 1994) and HSP47, which is a molecular chapernone (Wang, 1992).

Finally, several conclusions regarding regulation of uterine IEL were made using the unilaterally-pregnant ewe to test systemic and local effects of pregnancy on activation of endometrial $\gamma\delta$ $TCR^+$ IEL. First, $\gamma\delta$ $TCR^+$ IEL was functionally different from PBL with respect to expression of CD25, CD44, CD29 and Mel 14. Secondly, there was no difference in proportions of IEL staining positive for CD25, CD44, CD29 and Mel 14 between IEL from cyclic uteri and those from non-pregnant uterine horn of pregnancy. This was true despite the high secretion rate of UTM-proteins in the non-pregnant horn. In contrast, there was a higher proportion of uterine $\gamma\delta$ $TCR^+$ IEL from the conceptus-containing uterine horn that stained positive

for CD25, CD44, CD29 and Mel 14 than $\gamma\delta$ TCR$^+$ IEL from the non-pregnant uterine horn or cyclic uterus. These data provide evidence for activation of $\gamma\delta$ TCR$^+$ IEL by the local presence of the conceptus. Interestingly, this finding is consistent with the activation of uterine $\gamma\delta$ TCR$^+$ IEL induced by trophoblast in mice (Heyborne et al., 1994). Activated uterine T cells may produce immunosuppressive cytokines to regulate other populations of lymphocytes that potentially attack trophoblast (Suzuki et al., 1995). Local immunosuppressive factors including UTM-proteins do not appear to inhibit the uterine $\gamma\delta$ TCR$^+$ cell population during pregnancy.

Based on present findings, a hypothetical model is proposed to understand cellular mechanisms involved in protection of the sheep placenta from maternal immunological attack (Figure 7-2). According to this model, the trophoblast is at risk of lysis mediated by cytotoxic T cells (CTL), NK cells and LAK cells. Progesterone induces uterine epithelial cells to synthesize UTM-proteins, which block immunological attack of the trophoblast via CTL, NK and LAK cells. However, uterine $\gamma\delta$ TCR$^+$ IEL become activated by the presence of the conceptus even though there are locally-produced immunosuppressive factors including UTM-proteins. Activated $\gamma\delta$ TCR$^+$ IEL may also produce cytokines that may either 1) promote placental growth and hormone secretion or 2) inhibit activation and functions of other uterine lymphocytes as reported for TGF-$\beta$ secreted by mouse uterine $\gamma\delta$ TCR$^+$ cells.

Future work regarding UTM-proteins is required to study the relationship

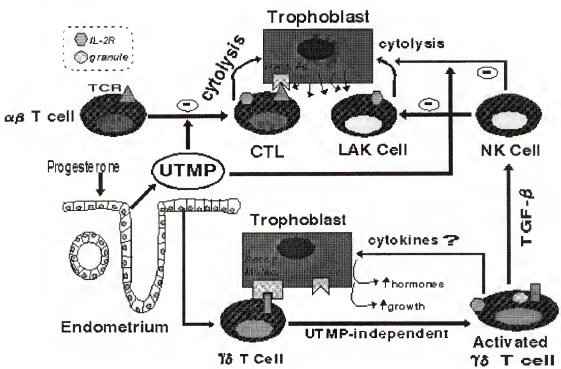


Figure 7-2 A modified model shows interactions between the maternal immune system and the conceptus. The model was originally proposed by Hansen and Liu (1996). Cytotoxic lymphocytes (CTL); Lymphokine activated killer (LAK) cells; Natural killer (NK) cells.

between the structure and function of UTM-proteins to elucidate functional domains of the proteins that are involved in immunosuppressive activity and to evaluate whether activity of UTM-proteins require the "stressed" confirmation characteristic of many serpins. Additional work is needed to identify receptors for UTM-proteins on uterine IEL and PBL and the signal transduction pathways induced by UTM-proteins. Such experiments may yield novel information on regulation of lymphocyte

function by serpins. Finally, the role of uterine $\gamma\delta$ $TCR^+$ IEL and other populations of uterine lymphocytes in pregnancy should be ascertained. Perhaps, these cells play a critical role in conceptus-driven changes in maternal immune function while UTM-proteins mediate effects of progesterone. One possibility that needs examination is whether the cells produce cytokines that stimulate placental development. Understanding the mechanisms by which the maternal immune system affects the development and differentiation of trophoblast may lead to practical strategies for altering immune function in a beneficial manner for survival and development of conceptus.

## APPENDIX A
## PROTOCOL FOR PURIFICATION OF MOUSE MONOCLONAL ANTIBODY BY FPLC

Materials

1. Protein A Column (Econo-Pac from Bio-Rad)

2. Washing buffer 1 (0.5 M Sodium phosphate, pH 7.4)

3. Washing buffer 2 (1.5 M Sodium phosphate, pH 8.0)

4. Binding Buffer (3 M NaCl, 1.5 M Glycine, pH 8.9)

5. Elution Buffer A (0.1 M Citric acid, pH 5.0)

6. Elution Buffer B (0.2 M Sodium phosphate, pH 7.8)

Preparation of the column

1. Set pump flow rate to 2.0 ml/min and keep pressure under 350 kpi, otherwise the column will be broken.

2. Wash the column with degassed 0.5 M Sodium phosphate for 10 min.

3. Wash the column with degassed 1.5 M Sodium phosphate for 10 min.

4. Equilibrate the column with binding buffer for 20 min at 2 ml/min and reduce the flow rate to 0.5 ml/min.

Sample preparation

Mouse ascites are diluted with the binding buffer. Dilution is dependent on sample, but commonly 1:10 dilution is used. If sample is serum-free supernatant,

further dialyze supernatant against binding buffer over night at 4 C.

Purification

1. Load 5 ml of sample on the column.

2. Wash the binding buffer at 0.5 ml/min of flow rate for 10 min.

3. Elute immunoglobulins with a gradient pH elution buffer from 7.8 to 5.0 for 10 min.

4. Neutralize the eluted samples immediately after elution with Tris-HCl (1 M, pH 8.8) or 1 N NaOH. Prolonged exposure of the purified immunoglobulin to acid pH should be avoided.

5.

Regeneration of protein A column

1. Regenerate the column with 25 ml of 50% methanol after each use at 0.5 ml/min of flow rate.

2. Equilibrate with 25-50 ml of 1.5 M sodium phosphate buffer if the column is to be used.

Care of the column

1. Wash the column with 25 ml of 50% methanol at 0.5 ml /min of flow rate.

2. Wash the column with 20-30 ml of 0.1 N NaOH.

3. Equilibrate the column with at least 25 ml of the binding buffer.

APPENDIX B

PROTOCOL FOR COUPLING BIOTIN HYDRAZIDE TO IMMUNOGLOBULIN

Materials

1. Labelling buffer (0.1 M sodium acetate buffer, pH 5.5)
   Glycerol.

2. Biotin hydrazide (Pierce)

3. Sodium meta-periodate solution (20 mM sodium periodate in the labelling buffer). Prepare fresh and keep in dark.

4. Antibody in the binding buffer.

Procedure

1. Take 1 ml of cold IgG solution (2 mg/ml) and add 1 ml of cold sodium meta-periodate solution into a test tube. Allow the oxidation reaction to proceed for 2 h at on ice in the dark.

2. Add glycerol into the tube to a final concentration of 15 mM and incubate for 5 min at on ice to stop the oxidation. Aternatively, immunoglobulin is direactly dialyzed against 0.1 M sodium acetate buffer (pH 5.5).

3. Dialyze the sample three times against labelling buffer for 4 h.

4. Add biotin hydrazide to the dialyzed sample to a final concentration of 10 mM or 1.2 mg/ml and allow reacting to proceed with mild agitation for 2 h at room temperature.

5. Dialyze biotinaylated samples versus a suitable buffer (PBS, pH 7.4, 0.02% azide) overnight at 4 C.

6. Store the biotinaylated sample at -20 C.

7. Test working solution concentration by using flow cytometry.

## APPENDIX C
## TWO COLOR FLOW CYTOMETRY

Materials

1. Staining buffer (0.1 M PBS + 1% (w/v) BSA + 0.02% Azide + 0.1 (w/v)% EDTA, pH 7.6)

2. 12 x 75 mm tubes (Polystyrene, round-bottom tube without cap)

3. Streptavidin-R-PE (Sigma)

4. Antimouse IgG (whole molecule) coupled to FITC

5. Biotinylated antibody.

6. 4% Paraformaldehyde in PBS.

7. Blocking buffer (10% normal heat-inactivated mouse serum in staining buffer).

Procedure

1. Prepare lymphocytes following the procedure for lymphocyte purification.

2. Divide 1-2 x $10^6$ cells/0.1 ml into a 12 x 75 mm tube

3. Cells are washed with staining buffer two times by centrifugation at 800 rpm for 5 min.

4. Cells are reacted with 100 $\mu$l antibody for 30 min at on ice.

5. Cells are washed twice in staining buffer by centrifugation, suspended in 100 $\mu$l of FITC-conjugated $F(ab')_2$ fragments of sheep anti-mouse Ig (determine experimently 1:200) and incubated for 30 min on ice. Cells are then washed as before.

6. Block free anti-mouse Ig binding sites by using blocking buffer for 30 min on ice. This step is not required for all antibody staining.

7. After washing, cells are incubated with biotinylated antibody conjugate for 30 min on ice.

8. The cells are washed in staining buffer again and 10 $\mu$l Streptavidin-R-PE is added into tube bottom for 30 min on ice.

9. The cells are washed again in staining buffer and then ready for flow cytometry. If flow cytometry is not to be performed immediately, the samples can be fixed in 100 $\mu$l 4% paraformaldehyde for 10 min on ice and then wash twice like previous steps, and kept in the dark.

## APPENDIX E
## $^{51}Cr$ RELEASE CYTOTOXICITY ASSAY

Materials

1. RPMI-1640 medium and complete modified RPMI-1640 medium containing 10% bovine calf serum, 100 IU/ml of penicillin, 100 $\mu$g/mg of streptomycin and 1 mM L-glutamine for preparation of lymphocytes. (Another optional medium shown to with for ovine lymphocyte proliferation assays is TCM199+10% horse serum, 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin, 1 mM L-glutamine and 5 mM 2-mercaptoethanol).

2. Histopaque-1077 and red blood lysis buffer (Sigma, catalog number 1077-1, R 7757).

3. Sodium Chromium-51, 1 mCi/ml (Sodium chromate in aqueous solution) for labelling target cells (Amersham Corporation, Catalog number-CJS.2V).

4. Sterile 15 ml conical centrifuge tubes for purification of effector cells.

5. 96-well V-shape-microplates for the release assay (Dytnatech Laboratories,Inc., Catalog No. 001-010-2701).

6. 1 ml sterile syringe for adding Sodium Chromium-51 to label target cells.

7. Target cells (YAC-1 cells for mouse assay; K562 cells for sheep assay)

9. Culture dishes (sterile 15 x 100 mm petri dish) or culture flasks (75 $cm^2$) to remove adherent cells (macrophages) from lymphocytes.

Procedure

Preparation of Sheep Effector Cells

1. Collect 50 ml sheep blood from jugular with VACUTAINER tube containing sodium heparin. This will provide enough cells for one 96-well microplate. Take out RMPI-1640 and Histopaque-1077 from cooler and allow warm them to room temperature before using.

2. Centrifuge blood for 20-30 min at 1500 rpm (400 X g) to obtain buffy coat.

3. Remove the buffy coat and mix with equal volume of RPMI-1640 in a sterile culture tube. Carefully place the mixed buffy coat layer on the top of an equal

volume of Histopaque-1077 and centrifuge the tube for 30 min at 400 Xg.

4. Transfer the white layer of leukocytes from the interface in the tube to another tube containing RPMI-1640.

5. If there is no contamination of red blood cells in the layer of leukocytes, wash the purified cells twice at 1000 rpm (IEC) for 10 min. This step will remove platelets which could otherwise interfere with the culture. If red blood cells are present in the leukocyte fraction, centrifuge it, obtain the pellet and then resuspend cells in red blood cell lysis buffer (5:1 ratio of buffer to pellet volume). After waiting for 10 seconds, add 10 ml RPMI-1640 to cell, wash and centrifuge.

6. Resuspend the pellet in 2 ml of RPMI-1640 supplemented with 10 % bovine calf serum and 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin and 1 mM L-glutamine or use TCM 199 for the culture.
7. Count cells with a hemocytometer following the lab manual and adjust cell concentration to 1 X $10^7$/ml.

8. To remove macrophages, culture cell suspension in a 100 X 15 mm petri dish and incubate at 37 C and 5% $CO_2$ for 3 h. Then pour cell suspension into a 15 ml centrifuge tube, spin at 1500 rpm for 10 min, pour off the supernatant, and resuspend the pellet in 2 ml of complete RPMI-1640 medium. Count cell number and viability, and adjust to 1 X $10^7$ live cells/ml as effector cells for cytotoxicity release assay.

Preparation of Mouse Effector Cells

1. Remove spleen at sacrifice and place in a 100 x 20 mm tissue culture dish containing 10 ml of incomplete modified RPMI-1640.

2. After trimming the connective tissues around the spleen, splenocytes are washed from the spleen by repeated injection of incomplete modified RPMI-1640 into spleen tissue.

3. The cells are wahsed twice in incomplete modified RPMI-1640 and adjusted to 2.5 x $10^7$ cells/ml.

4. Aliquots (2 ml of cell suspension) are placed on the top of an equal volume of Histopaque 1077 in a 17 x 100 mm culture tube and centrifuged at 400 x g for 30 min.

5. Cells at the interface are collected, washed twice in modified in modified RPMI-1640, resuspended to 1 x $10^7$ cells/ml in complete modified medium and

cultured for 1 hr at 37 C and 5% $CO_2$ in a 100 x 15 mm petri dish.

6. The cell suspension containing non-adherent cells is collected, centrifuged at 400 x g for 10 min to remove supernatant, and resuspended in complete modified medium to a concentration of 5 x $10^6$ cells.

Labelling Target Cells

1. Target cell lines (K562 for sheep effector cells or YAC-1 for mouse effector cells) are continuously cultured in 10 ml of complete RPMI-1640 medium in a 25 $cm^2$ flask at 37 C and 5% $CO_2$. The cells are subcultured twice weekly. To do this, centrifuge cell suspension in a sterile tube at 1500 rpm for 10 min and pour off the supernatant. Resuspended pellet with complete RPMI-1640. In general, one culture flask can be divided into three culture flasks, when cell concentration is about 1 x $10^6$/ml.

2. Collect the cells before labelling by centrifugation at 1500 rpm for 10 min. Wash the cells twice in complete RPMI-1640 medium by centrifuging at 1500 rpm for 10 min and resuspend the pellet in 2 ml of medium.

3. Stain 50 $\mu$l of the cell suspension with equal volume of trypan blue to examine cell viability and count cell number with hemacytometer.

4. Adjust cell concentration to 1 X $10^6$ live cells/ml of complete RPMI-1640 and centrifuge a 1 ml aliquot of cells at 1500 rpm for 5 min.

5. Remove 0.9 ml of the supernatant, add 100 $\mu$l of $Na_2{}^{51}CrO_4$ and gently shake the tube several times to disperse the cells and mix with the labelling substance on the bottom of the tube.

6. Incubate the mixture at 37 C and 5% $CO_2$ for 60-90 min and then wash the labeled cells in complete RPMI-1640 medium three times by centrifuging at 1500 rpm for 10 min. Be careful to disposal of all supernatant as radioactive waste.
7. Resuspend the pellet in 10 ml complete RPMI-1640 for the cytotoxicity assay.

$^{51}$Cr-Release Assay

1. Add 100 $\mu$l of effector cell suspension to wells of a 96-well V-shape microplate in triplicate. For these wells, the ratio of effector to target (E:T) will be 100:1 for sheep and 50:1 for mouse when target cells are added to the wells.

2. Prepare other E:T ratio by using two-fold serial dilutions of effector cells to

make dilutions in wells of 96 well plates.

4. Add 100 $\mu$l of target cell suspension (containing 1 X $10^4$) to each well.

5. All assays should contain two controls: spontaneous release and maximum release. Spontaneous release represents dpm from culture containing 1 X $10^4$ labelled target cells in 200 $\mu$l of the medium. Maximum release represents dpm from a mixture of 100 $\mu$l 1% (v/v) Triton X-100 and 100 $\mu$l medium containing 1 X $10^4$ labelled target cells.

6. After adding cells, in order to facilitate conjugate formation, centrifuge plate(s) at 150 X g for 5 min. Then incubate the plate(s) at 37 C and 5% $CO_2$ for 20 h (sheep) or 4 h (mice).

7. Centrifuge plate(s) at 300 Xg for 10 min and pipette 100 $\mu$l of supernatant into 12x75 mm culture tube.

8. Count tubes for radioactivity in a gamma scintillation counter.

9. Calculate % specific release=(sample dpm - spontaneous dpm)/(maximum dpm -
spontaneous dpm).

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## BIOGRAPHICAL SKETCH

Wen-Jun Liu was born March 6, 1959, in Tianjing, a coastal city in the Northern of China, as the third son of Zongyin Liu and Guangxia Lu. Before coming to college, he dreamed of becoming a chemist. However, his interests subsequently changed and he obtained the Doctor of Veterinary Medicine degree from the College of Veterinary Medicine at Beijing Agricultural University in 1982. After graduation, he entered the Graduate School of the Beijing Agricultural University under the supervision of Dr. Baoren Li of the Department of Anatomy, College of Veterinary Medicine. His thesis research concerned the development of the blood-testis barrier and spermatogenesis in the pig. He received the Master of Science Degree in 1985. From 1985 to 1991, Dr. Liu served as a faculty member at the Beijing Agricultural University, working in laboratory animal medicine. He married Shenyun Mou in 1987. One year later, he became a father when his lovely daughter, Tong Liu, was born. Dr. Liu served as an Assistant Director of the Institute of Laboratory Animal Medicine at Beijing Agricultural University from 1987 to 1990 and was promoted to research professor in 1990. His research interests at Beijing were broad and involved research on pigs, mice and chickens. His major interest was the study of immunobiology of the mini-pig and use of the pig as an animal model in

cardiovascular biology and organ transplantation studies. His colleagues and he translated "Diseases of Poultry" edited by B.W. Calnek and "Diseases of Swine" edited by A.D Leman into Chinese.

After a short period at Institute of Endocrinology of Gunma University as a Ph.D. student who received Japan Government Scholarship in Japan, Dr. Liu moved to the University of Florida in 1992 to begin work torwards the Ph.D. degree under the supervision of Dr. Peter J. Hansen. His research focused on interactions of lymphocytes and uterine immunosuppressive proteins. While at Florida, he was elected to membership in the Sigma Xi honorary society. Also, his wife and he had a little Gator, his son, David Liu, who was born November 15, 1995, in Gainesville.

After finishing his Ph.D., Dr. Liu will start a postdoctoral fellowship with Dr. Rodger McEver at the Department of Medicine, University of Oklahoma. Here he plans to continue his long term studies in immunology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Peter J. Hansen, Chairman
Professor of Animal Science

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E. Paul Gibbs
Professor of Veterinary Medicine

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Lynn H. Larkin
Professor of Anatomy and Cell Biology

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Frank A. Simmen
Professor of Animal Science

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William W. Thatcher
Graduate Research Professor of Animal Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1996

Jack L. Fry

Dean, College of Agriculture

Dean, Graduate School